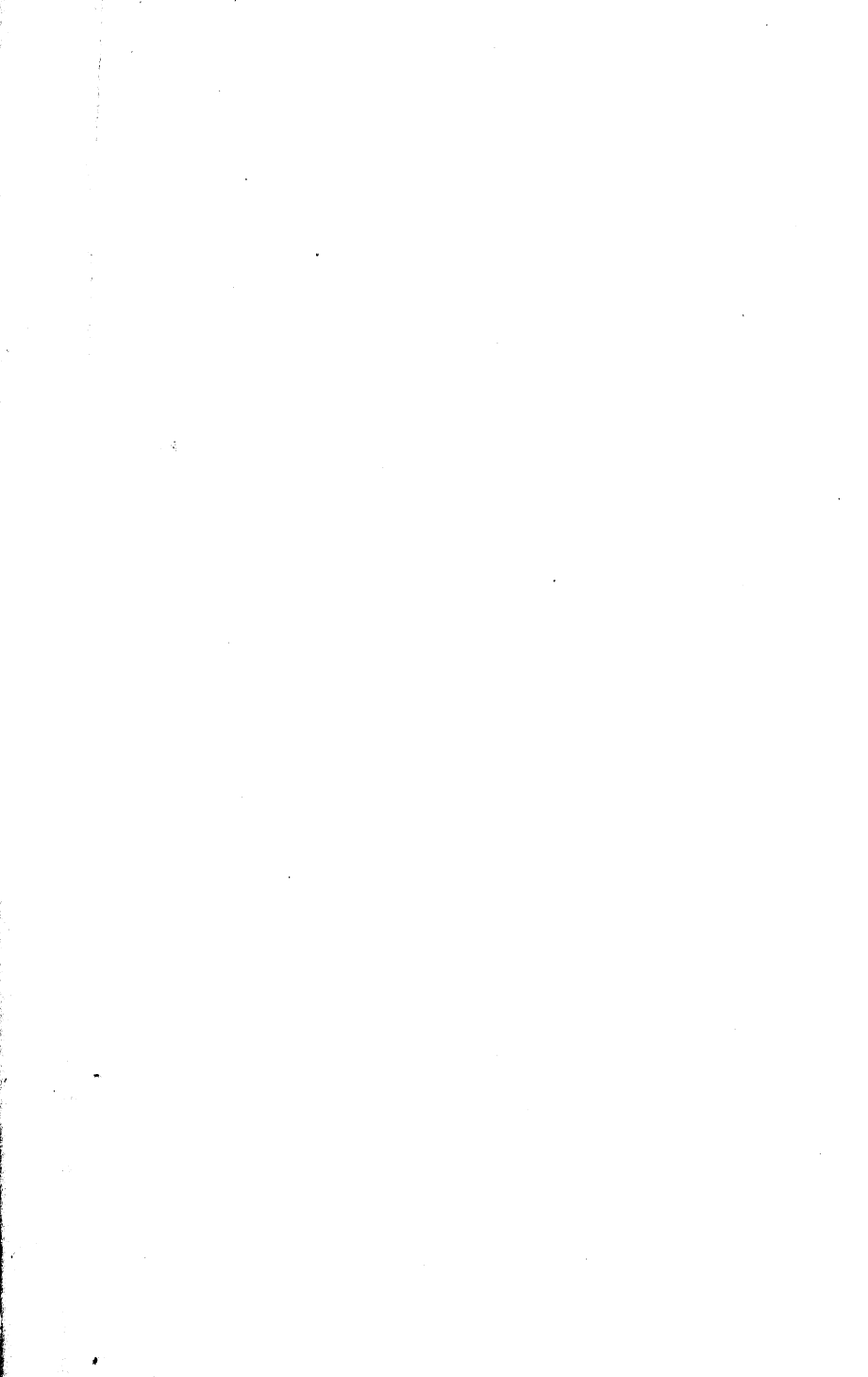




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STAIN TECHNOLOGY

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ISOPROPYL ALCOHOL IN THE PARAFFIN INFILTRATION TECHNIC

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ABSTRACT.—A method is described for using isopropyl alcohol for dehydration of animal tissues preceding melted paraffin infiltration. Advantages of the technic are: simplicity, low cost, low toxicity, and diminished distortion and hardening of the tissues.

Isopropyl alcohol has been used for many years in histological technic as a substitute for ethyl alcohol to dehydrate tissues; see Griffin (1922), Bradbury (1931), Herman (1941). These authors all reported that isopropyl alcohol is either equal or superior to ethyl alcohol in performance. Reasons motivating the substitution are that isopropyl alcohol is readily available at low cost, and has been found to shrink and harden animal tissues less. Isopropyl alcohol does not have the undesirable toxic effects of dioxane; as it is unpalatable, it is free of internal revenue restrictions.

In 1929, Sheridan (1929) published his technic of using normal propyl alcohol and low melting point paraffin in paraffin infiltration. The method of using isopropyl alcohol, herein described, is quite similar. The substitution of isopropyl alcohol for normal propyl alcohol is made because of its low cost and ease of acquisition.

The published technics have not included the direct transfer of tissues from isopropyl alcohol to melted paraffin, but used an intermediate paraffin solvent or clearing agent. This intermediate step, which may cause hardening and distortion, is unnecessary. While cold paraffin is nearly insoluble in isopropyl alcohol, melted paraffin is completely miscible. Because of the low boiling point and low specific gravity of isopropyl alcohol, it is readily and completely expelled from both the tissues and the paraffin baths.

The schedule given below is one particularly adapted to the

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author's working hours. For the working conditions in other laboratories a modification of the schedule might be advisable. Formalin-fixed animal tissues are washed overnight to remove the fixative. They are dehydrated, infiltrated and embedded as follows:

- (1) Isopropyl alcohol, 60%.....1 hr.
- (2) Isopropyl alcohol, 99%.....1 hr.
- (3) Isopropyl alcohol, 99%.....2 hr.
- (4) Isopropyl alcohol, 99% (40-45°C.).....3 hr.
- (5) Melted Tissuemat (M.P. 50-52°C.).....1 hr.
- (6) Melted Tissuemat (M.P. 50-52°C.).....Overnight
- (7) Embed and block in tissuemat of right melting point to suit cutting conditions.

After using this embedding technic, tissue sections 8μ thick are easily prepared and differential staining is not impaired.

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DIFFERENTIAL IN TOTO STAINING OF BONE, CARTILAGE AND SOFT TISSUES

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ABSTRACT.—The following method for staining bone and cartilage allows study of the gross cleared specimen and does not injure the tissues for subsequent microscopic study: Fix in 10% neutral formalin; bleach thoroughly in 3% H_2O_2 in sunlight. Wash in distilled water. Stain bone 24 hours in 0.01 g. of Biebrich scarlet in 100 ml. of distilled water. Destain in 95% alcohol until soft tissues and cartilage are colorless. Stain cartilage 24 hours in a pH2 buffer solution of 2.1g. of citric acid per 100 ml. of water with 0.001 g. of methylene blue. Destain in pH2 buffer solution until soft tissues are pale. Dehydrate in two changes of 95% alcohol in preparation for clearing. (This completes the destaining and may remove too much stain from the cartilage if destaining in the pH2 solution has been carried too far.) Place in Groat's clearing fluid and cover loosely so that the alcohol may evaporate, or remove the alcohol in vacuo. Groat's Mixture No. 19 is usually satisfactory.

For a combined stain, first stain bone, as above, and then apply the cartilage stain.

Seal jars with an ordinary liquid wood glue such as LePage's.

INTRODUCTION

In a study of fetal skeletal deformities which the author was undertaking, it soon became apparent that it would be advantageous first to study the specimens grossly with the bone and cartilage stained and the soft tissues transparent, and then to follow this with microscopic examination of the same specimens. This paper presents methods of selective *in toto* staining of bone and cartilage, which when combined with Groat's (1941) method of clearing, do not damage the tissue for subsequent microscopic study.

REVIEW OF THE LITERATURE

Clearing methods: Schultze's method of clearing as described by Hill, (1906) depends upon maceration of the soft tissues in 1% KOH and has the disadvantage of making subsequent microscopic study impossible.

¹This work was done in the Department of Anatomy of the University of Wisconsin while the author was an Orthopedic Resident in the Department of Surgery

Several clearing methods are based upon the principle of immersing the specimen in a liquid with a refractive index similar to that of the soft tissue of the specimen. Spalteholz (1914) recommended methyl salicylate and Lundvall (1904) used carbon disulfide. Groat (1941) achieved maximum clearing by adjusting the refractive index of a mixture of triorthocresyl phosphate and tributyl phosphate to that of the tissue.

Bone stains: Schultze's alizarin bone stain, as described by Hill (1906), depends upon maceration of the soft tissues with KOH until they no longer take the stain. Batson (1921) found a number of dyes suitable for Schultze's method. Williams (1941) combined a cartilage stain with Schultze's bone stain.

Cartilage stains: Lundvall (1904) described a cartilage stain with an acidified alcoholic solution of toluidine blue. He credited the stain to Van Wijhe. Da Rocha (1917) described additional dyes for this stain. Miller (1921) used the KOH method of clearing with this method. Hamberger (1940) described a similar stain using methylene blue.

EXPERIMENTAL WORK

Buffer solutions from pH2 to 11 at intervals of 1 pH unit were made up by the methods of McIlvaine and of Kolthoff and Vlesschhower as given by Clark (1928). Dyes were tested in the buffer solutions and in distilled water. Alcohol, acetone and acid alcohol were tried in destaining. Test objects were split vertebrae of 10 cm. fetal pigs fixed in neutral formalin and bleached in H_2O_2 .

Bone stains: A group of dyes classed as "acid" by Conn (1940) stained bone differentially only in distilled water (pH6). Destaining was done in 60% to 95% ethyl alcohol. There was one exception: fast green FCF stained bone not only at pH of distilled water (pH6) but throughout the whole range to pH11. Since this dye is an indicator the stain was green at pH6 and blue at pH11. Because all bone stains were slightly transparent they were less striking than Schultze's alizarin stain.

Cartilage stains: A group of dyes classed as "basic" by Conn (1940) stained only cartilage at pH2. Destaining was best in citric acid in water at pH2, or in 60% to 95% alcohol. In distilled water a fast stain of all tissues occurred. There was no stain at pH11. Cartilage could be successfully stained following the bone stain, but if the cartilage stain was performed first the bone stain produced a fast stain of all tissues, as though it had taken place at pH2. Attempts to wash out or neutralize the acid used in the cartilage stain were unsuccessful.

STAINING METHOD

Preparation of specimens: Always remove the liver since it is almost impossible to bleach and its pigment darkens the clearing fluid. The staining and destaining process depends upon diffusion of solutions through the tissues and is facilitated by complete evisceration and by opening the cranial cavity through the fontanelles. A sagittally split specimen is excellent and demonstrates the skeleton without overlap. In large specimens incisions into large muscle masses such as occur in the neck facilitate diffusion.

Fixation and bleaching: Fix well in 10% formalin made neutral by addition of an excess of calcium carbonate. The presence of traces of formic acid interferes with the bone stain. Bleach thoroughly in several changes of 3% H_2O_2 in distilled water in sunlight. This may take several weeks. Wash 24 hours in distilled water. Bubbles in the tissue may be removed in vacuo or with a needle and syringe.

Concentration of dye used: The optimum dye concentrations are given in Table 1. Deviations from the optimum concentration of about $5\times$ may produce a pale stain or make destaining difficult; e.g. orange II 0.01%—pale; 0.05%—sharp; 0.1%—soft tissue difficult to destain. In general more mature specimens require a more dilute bone stain and a more concentrated cartilage stain.

General: The timing and concentrations of dyes listed below are for an eviscerated 10 cm. pig fetus.

Repeated staining and destaining destroy the affinity of the tissues for the dye.

Bone stain: Stain 24 hours in a solution of any one of the dyes made up according to Table 1. Biebrich scarlet is very good.

Destain in alcohol (60% to 95% is suitable) until soft tissues and cartilage are colorless. The color shining through from underlying bone, particularly over the long bones, may lead to excessive destaining and should be disregarded.

Cartilage stains: Stain 24 hours in a solution of any one of the dyes made up according to Table 1. Methylene blue is very good.

Destain in pH2 buffer solution (21 g. citric acid per 1 liter distilled water) until soft tissue is pale. The final dehydration with 95% alcohol in preparation for clearing will complete the destaining and may remove too much stain from the cartilage if destaining in the pH2 is overdone.

Combined stain of bone and cartilage: Use the bone stain first, and, when destaining is complete, wash in a few changes of distilled water until the specimen sinks. Then stain for cartilage and clear.

Clearing: After staining dehydrate the specimen in two changes of 95% alcohol. Place in Groat's clearing fluid and cover loosely so that the alcohol may evaporate, or remove the alcohol in vacuo. Groat's mixture No. 19 is usually satisfactory.

Fluorescent dyes: Fluorescent dyes aid in the study of surface contours, since visible fluorescence occurs almost entirely at the surface of the fluorescing object or solution. Direct or oblique illumination

TABLE 1.—THE AMOUNT OF DYE AND CITRIC ACID TO USE PER LITER OF DISTILLED WATER IN MAKING UP THE BONE AND CARTILAGE STAINING SOLUTIONS.

Some of the characteristics of the stains are also given.

DYES	G. per liter	Citric acid G. per liter	Color of stained tissue	Rela- tively fast stains*	Fluoresces under "glass" ultra violet	Soft tissues difficult to de- stain
BONE STAINS						
Benzopurpurin (G)	0.1	—	Red			
Biebrich scarlet (NA)	0.1	—	Red	+		
Bordeau red (G)	0.1	—	Red	+		
Eosin Y (NA)	0.1	—	Orange		+	
Fast green FCF (C & B)	0.1	—	Green†	+	+	
Orange II (NA) (Cert. NOB-2)	0.5	—	Yellow	+		
Ponceau red (NA)	0.1	—	Red	+		
CARTILAGE STAINS						
Cresyl echt violet (NA)	0.02	21	Blue		+	+
Gentian violet (G)	0.02	21	Blue			+
Janus green (NA) (Cert. NJ-1)	0.02	21	Blue			
Methylene blue (NA)	0.01	21	Blue			
Nile blue (NA)	0.02	21	Blue	+		+
Thionin (G)	0.02	21	Blue			+
Toluidin blue (G)	0.02	21	Blue			
Victoria blue (G)	0.02	21	Blue			+

*Specimens stained by those indicated as fast had not faded appreciably after three months of exposure in a sunny window. The others fade slowly if exposed to light. With a glass electrode the bone stain solutions prove to be pH 6.0 ± 0.2 , and the cartilage stain solutions pH 2 ± 0.1 .

†Fast green will produce a blue stain if made up at pH 11 by buffering with 5.3 g. of Na_2CO_3 per liter. Sources of dyes are indicated as follows: (C & B) Coleman and Bell; (G) Gruebler; (NA) National Aniline & Chemical Co.

must of course be used instead of transmitted light. Suitable sources of near or "glass" ultra-violet are sunlight, or any white burning or overloaded electric light, such as a focusing microscope lamp or a flashlight with new batteries. True ultra-violet is unsuitable since it causes all tissue to fluoresce white, even though it is cleared and not stained.

Fluorescent bone stain: A brilliant fluorescent bone stain is produced by staining 24 hours in a saturated alcoholic solution of fluorescein

(Gruebler) and destaining with alcohol. This is unsuitable for combined stains as it destains during cartilage staining. The stain also fades rapidly if exposed to sunlight.

Fluorescent soft tissue stain: Stain with eosin Y, 1 g. and citric acid, 21 g. per liter H₂O (distilled). Destaining is not necessary. Surface fluorescence in cleared specimens from tendons, blood vessels and nerves outlines their course when examined by reflected light.

Demonstration of epiphyseal lines by the cartilage stains: In specimens from adolescents only the epiphyseal lines and a thin layer of cartilage surrounding the epiphysis stain. The growing cap of cartilage on osteochondromas stains similarly. In fetal specimens decalcification with 1% HNO₃ reduces the affinity of cartilage for the dye except at the epiphyseal plate. Prolonged HNO₃ treatment destroys the staining quality of all cartilage. This varies greatly with the maturity of the specimen, so no definite treatment time can be specified. Adult articular cartilage does not stain by this method.

Unsatisfactory stains: Stains tried and found to be unsatisfactory for these methods are the following: Acid fuchsin, acid green, acriflavine, alizarin red S, amaranth, anilin blue W.S., aurantia, azure A, basic fuchsin (magenta red), Bismarck brown, carmine, chrysoidin (Grübler), dianil blue, "erythrosin pur" (Grübler), fast acid blue, hematein, iodine green, malachite green, mercurochrome, methyl green, methyl red, naphthaline blue, nigrosin, nitrazine, orange G, safranin, Sudan IV (scarlet red), Trypan blue.

Sealing specimen jars: Since Groat's clearing mixture is not volatile, it is not essential to seal jars. Ordinary LePage's wood glue is the best sealing agent for this solution we have found. The surfaces to which the glue is applied must be cleaned and dried with acetone or some similar solvent of the clearer, and the clearing agent must be kept from contact with the glue until it has dried. The usual sealing compounds are dissolved by the clearing solution.

Sectioning subsequent to in toto clearing: Tissues from stained specimens cleared by Groat's method may be infiltrated with paraffin directly from the triorthocresyl and tributyl phosphate mixture. Ordinarily it would probably be more satisfactory to wash out the clearing agent with some more volatile substance such as dioxane or cyclohexanone before placing the tissue in paraffin. Sections must be restained, as the processing removes most, if not all, of the *in toto* stain.

DISCUSSION

This staining method is presented on a purely empirical basis. Time did not permit further exploration of the problem or application

of the method to microscopic sections. The work of Silver (1942) with silver stains indicates that the isoelectric point of the various proteins is the determining factor in selective staining. Under high dry magnification the cartilage stain appears to be in the matrix, since rows of clear spaces corresponding to the cartilage cell columns were seen. There was a clear area 20 to 30 μ wide between the cartilage and the stained bone. Whether the bone stain depends upon the presence of calcium could not be determined since decalcification changed the specimen so that bone, cartilage and soft tissue stained.

SUMMARY

A method of *in toto* staining of fetal bones and cartilage which allows subsequent microscopic study of the specimen is presented. Control of pH is essential to the success of the method. Fluorescent stains for bone, cartilage and soft tissue are given, and also a method of staining epiphyseal plate cartilage. Groat's method of clearing is recommended.

ACKNOWLEDGEMENT

The author wishes to thank Dr. H. W. Mossman for suggesting this problem and for his constant help during its development.

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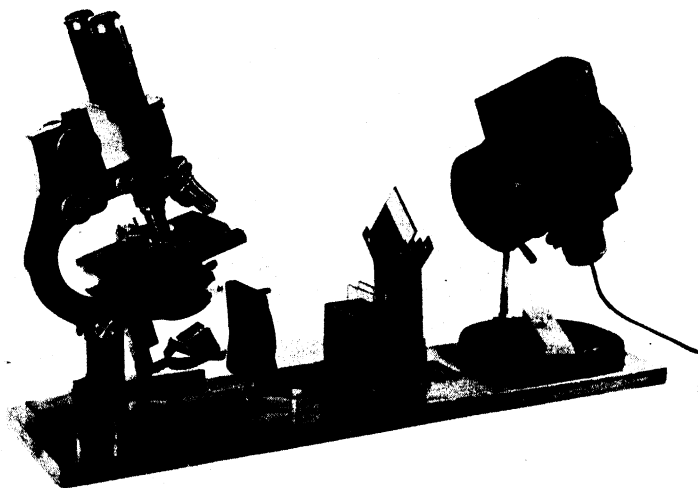
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A UTILIZATION OF THE KOHLER SYSTEM OF ILLUMINATION

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The following system of microscope lighting is based on ideas gleaned from a number of sources, particularly Belling (1930) and Shillaber (1944). The principles are not new and this article is primarily concerned with an implementation of the well known Kohler method. The chief points to be emphasized are the use of a prism and a polaroid variable density screen.

In lieu of an optical bench the components are mounted on a board. The light source is a six volt ribbon filament lamp in a housing equip-



ped with condensing lenses and an adjustable diaphragm. If a silvered mirror or other reflector is located in the lamp, it should be removed so that the only effective illumination is directly from the ribbon filament. The lamp is located about fifteen inches from the substage mirror.

In the middle of the board is a filter-holding rack made of small pieces of angle iron inserted in slots cut into the support. Next to it is a filter-holding box, built to hold filters made of 2×2 inch Wratt-

ten gelatin cemented between 2×3 inch microslides. The lips of the compartments are lined with velvet removed from discarded film packs. The filters are thus dusted and protected from light when inserted in the box. Wratten filters 61, 57A and 66 are used by the writer.

Immediately in front of the substage mirror is mounted a polaroid variable density screen. Any device by which one polaroid element can be rotated in reference to another will serve the purpose. The writer used a variable density screen removed from an Army Air Force gunsight¹ that ordinarily is mounted permanently in the line of vision of pursuit pilots by means of four bolts. Disassembly of the gunsight and judicious use of a hack saw will remove the variable screen with its hinges and two of the bolt mountings. Thus, the screen may be swung out of the optical axis to obtain maximum lighting.

A first surface mirror or a prism is very desirable in removing the secondary light images formed by the first surface of the ordinary mirror. Here, an air surfaced 90° prism is mounted, by use of Clarite, on the plane mirror. The silver of the mirror now serves as a part of the prism, converting it to a "silvered" prism that requires no further mounting. The entrance surface of a silvered prism need not be at perfect right angles to the axis of the light source to give good light reflection. Consequently, the microscope may be tilted as desired without marked disadvantage. The size of the reflecting surface of the prism is 34×23 mm. If a prism of less than 34 mm. length is used, it should be mounted higher on the mirror, since the optical axis of most microscopes meets the tilted mirror above its swinging axis. An alternative would be to follow Belling's suggestion (1930, p. 138) and shorten the stem of the mirror fork.

OPERATIONAL PROCEDURE

(1) Open diaphragm of lamp and focus the image of the incandescent ribbon filament on the diaphragm of the substage condenser. This need not be repeated except for occasional checking. Close diaphragm to about one half inch diameter.

(2) Bring material on slide into focus.

(3) Adjust the substage condenser until edges of the lamp diaphragm are in focus. Open the lamp diaphragm to just beyond the visual field. (N. B. If it is desired to illuminate the entire field of a 16 mm. objective by the Kohler system, the lamp will usually need to

¹Obtained from Edmund Salvage Co., Audubon, N. J. (Cat. No. 908-W).

be less than 15 inches from the mirror. The writer switches to diffuse lighting by placing a frosted glass in the filter holder.)

(4) Adjust the condenser diaphragm to best *resolution*, avoiding glare in one extreme and diffraction bands in the other.

(5) Adjust the polaroid elements to obtain a satisfactory *intensity* of light.

(6) Insert filters for desired contrast and readjust polaroid settings.

In principle the condenser diaphragm should be used only to adjust the cone of light from the condenser to the numerical aperture of the particular objective in use. The *intensity* of light is then regulated by other means; by neutral filters, or, in this case, by polaroid. The latter point is of importance in photomicrography. Changes can be made in the amount of transmitted light without altering the quality of the image.

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AN ADAPTABLE STAINING SCHEDULE FOR PLANT TISSUES

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ABSTRACT.—A general schedule for staining meristematic, maturing, and mature plant tissues is described. Treatment with a dilute aqueous solution of Delafield's hematoxylin is followed with staining in 0.1% safranin in 60% alcohol. Destaining of safranin may be partly accomplished in alcohol and completed by counterstaining with dilute fast green FCF in a xylene and alcohol mixture. Various modifications and adaptations are briefly discussed.

Preparation of good-quality research or teaching slides from paraffin-embedded tissues requires tedious and time-consuming manipulation when the usual staining methods are employed. In the course of several years various modifications of previously reported methods have been used and combined. The result has been the development of a schedule which is fairly rapid without sacrifice of permanency and sharp differentiation, and by which destaining problems are reduced to a minimum. In addition, the schedule has proved to be quite adaptable and has produced good results with a wide range of plant tissues. Differentiation of cell contents, nuclei, and cell walls is sharply produced in apical meristems, embryos, and other tissues which have been killed and fixed, at various stages of development, in formalin-acetic-alcohol and in Karpechenko's solutions (Rawlins, 1933).

After removal of paraffin from the sections with xylene, the slides are placed in absolute alcohol for a few seconds. They are then removed, the backs are wiped dry, and the alcohol is allowed to evaporate from the sections. Better results can be secured if, as the alcohol has evaporated, the same procedure is repeated with fresh alcohol. Contrary to the usual notion that once the paraffin has been removed the sections must not be allowed to dry, this procedure appears to be safe for plant tissues. Virtually complete dehydration of the tissues has already occurred during prior stages of preparation. Also, there is the advantage, in allowing the sections to become dry at this stage, that no trace of xylene is carried through the remainder of the series. The slides are then transferred to a Coplin jar of 95% ethyl alcohol and are ready for the following general schedule:

¹Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

- (1) Water rinse. (May include a mordant).
- (2) Delafield's hematoxylin (stock solution diluted to about 10% with distilled water), 5 to 15 minutes according to requirements. Avoid overstaining.
- (3) Water rinse (or differentiating agent followed by water rinse).
- (4) Safranin O, 0.1%, in 60% ethyl alcohol (addition of a few drops of 5% aqueous solution of sodium acetate will greatly improve the staining quality of the safranin solution). This dilution of safranin considerably minimizes the problems of overstaining and destaining. Some tissues are sufficiently stained in half an hour; others require at least two hours. The procedure may be hastened by use of warm solution in a paraffin oven for 5 to 15 minutes. Or, if it is more convenient to do so, sections may be left overnight or longer in the solution (at room temperature) without becoming too heavily stained.
- (5) Water rinse.
- (6) Destain in alcohol. (If necessary, a drop or two of 1 or 2% hydrochloric acid may be added to the alcohol. Destaining is slower in 95% alcohol than in more dilute solutions.)
- (7) Fast green serves as a destaining agent as well as a counterstain, and is easily controlled in the described solution. A few ml. of a saturated solution of fast green FCF in clove oil and alcohol (1:1) are added to a Coplin jar containing 50 ml. xylene and alcohol in a ratio of about 3:1. Generally, 1 to 3 minutes is sufficient to complete the destaining of the safranin to a desired stage and also to provide a good counterstain. The safranin staining may be adjusted according to results obtained with fast green.
- (8) Xylene-alcohol rinse (two different Coplin jars).
- (9) Two changes in xylene before mounting in balsam or a suitable synthetic resin.

This schedule may be modified by the use of Foster's (1934) technic with tannic acid and ferric chloride, in place of Delafield's. Some objection may be found to the results of the tannic acid and ferric chloride method, in that the ferric deposits in the middle lamellae and adjacent wall regions may be too heavy; thus the finer details, which are well differentiated with Delafield's technic, may be obscured. A part of this difficulty can be overcome with tannic acid and ferric chloride in solutions of 0.1%. About one minute in each solution generally is sufficient to define sharply the walls of meristematic cells, but the sections should be thoroughly rinsed after immersion in tannic acid solution and before they are placed in ferric chloride.

Delafield's formula also may be used following the tannic acid and ferric chloride treatment which serves additionally as a mordant. Several other hematoxylin preparations and mordants described by Cole (1943) also have been used in this schedule with excellent results. In fact, hematoxylin preparations, including alcoholic as well as aqueous solutions, can be manipulated to produce black nuclei, bluish

cytoplasm, and lavender cellulose walls; or (when combined with safranin and fast green) black or pink nuclei with deep red nucleoli, green or blue cytoplasm, lavender, or black cellulose walls, and red lignified walls can be obtained.

Northen (1936) has described several modifications of Foster's method which in part are also adaptable to the above schedule. Sharman (1943) has described a schedule using very dilute safranin in combination with orange G, tannic acid, and iron alum. Boke (1939) and Cross (1937) previously have reported methods using Delafield's preparation and fast green in a manner similar to those described here. Boke used safranin in xylene and alcohol with good results. The use of warm safranin for plant tissues has been previously reported by Shapiro (1947).

The combination of both hematoxylin and fast green with dilute safranin, as described here, provides a schedule of greater adaptability than do most methods. Structures containing closely associated meristematic, maturing and mature cells may be stained with uniformly good results for the different sorts of tissue present. In addition, the more delicate structures are well defined without loss of detail and may be easily photomicrographed under proper conditions of illumination.

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A NEW STAIN MIXTURE: ACETO-ORCEIN-FAST-GREEN

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ABSTRACT.—A new mixture consisting of aceto-orcein, fast green, and salt is described. The mixture provides contrast between nuclear material, nucleoli and cytoplasm without differentiation. We have found it most useful in the staining of teased tissues, squashes, smears and suspensions (fixed and unfixed). We recommend it for the great speed and simplicity with which it provides a stain having the contrast of much more laborious histological technics.

Aceto-orcein (La Cour, 1941) has for some time been a favorite stain of the cytologist. Its simplicity and speed, especially for fresh material, have made for its great popularity. However, when counterstaining is desired, it has been the practice to make a permanent preparation of the aceto-orcein stained preparation before staining with fast green. In an effort to preserve the advantages of speed and simplicity, we have developed a dye mixture for contrast staining.

The mixture is prepared from materials usually on hand in the laboratory:

1% orcein in 45% acetic acid ²	27 ml.
1% fast green in alcohol	3 ml.
2M NaCl.....	2 ml.

The greenish-purple mixture is applied to the preparation for a few minutes, covered with a cover slip, and examined (excess stain may be removed by light pressure over the cover slip with filter paper). Chromatin stains brownish-red, nucleoli and cytoplasm green. Thus, a preparation comparable to that obtained with the Feulgen stain counterstained with fast green is obtained in a few minutes, and with only one staining operation. The method is particularly applicable to teased tissues which are not readily fixed to the slide.

The addition of salt is essential for uniform staining. It was found, from experiments with fast green, that fast green stained much more intensely in the complete absence of salt than in its presence (Kurnick, 1947a). As a result, if material suspended in a salt-free medium

¹This work was done in part under an American Cancer Society fellowship (N. B. Kurnick) recommended by the Committee on Growth of the National Research Council.

²Two samples of orcein were studied: a) Grüber and (b) Coleman and Bell, crystallin, C. P. The latter sample, perhaps because of its less ready solubility, stains somewhat less intensely and the stained chromatin is of a browner shade than is obtained with the Grüber stain. Otherwise entirely similar results were obtained. The Fast Green FCF used was manufactured by National Aniline, Cert. No. NGf8.

(as acetic-alcohol) is stained with the aceto-orcein-fast-green mixture from which *salt* has been *omitted*, the characteristic differential staining is obscured by over-all dense staining with the fast green. The addition of salt to the stain itself has obviated this difficulty.

We have applied this stain mixture to fresh teased onion root tips and to onion root tips preserved in acetic alcohol. They stain alike with contrast as described above: brownish-red chromatin, green nucleoli and cytoplasm. Fresh and fixed grasshopper testes likewise stain similarly. Here again, a remarkable similarity to the Feulgen stain (counterstained with fast green) is noted in that immature sperm cells stain with the usual contrast between brownish-red chromatin and green nucleoli and cytoplasm, and the mature spermatzoa appear as reddish filamentous heads with pale green tails. Connective tissue stains pale green. Nuclei isolated from beef thymus and liver by the citric acid technic (Stoneburg, 1936) and suspended in citric acid, saline, or acetic-alcohol all demonstrated the expected staining characteristics with the mixture. We have found it very useful in checking on the purity of isolated chromosomes (Mirsky and Ris, 1947) since the chromatin threads reveal no green staining, while nucleoli, contaminating cytoplasmic and connective tissue debris stain green. Paraffin sections of mammalian organs have been passed through xylene, and alcohol to water, covered with the stain mixture for five minutes and then examined. Again the characteristic differentiation between chromatin and other structures is obtained. The chromatin stains brownish red; cytoplasm, nucleoli, collagen, and erythrocytes, green. Satisfactory permanent preparations were obtained by removing the cover glass and dipping the slide successively in 70% alcohol, 95% alcohol, 100% alcohol, xylene and mounting in Clarite. The alcohol removes some of the orcein, but enough is retained by the chromatin to provide a satisfactory stain while the green of the other structures stands out even more brilliantly (with the overlying layer of stain washed away).

Thus, the aceto-orcein-fast-green-salt mixture provides a simple stain mixture which gives contrast between cellular structures without washing or differentiation. The stain should find its greatest use in fresh teased material, squashes, smears and suspensions of tissues in fluid.

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A RAPID STAIN METHOD FOR DETECTING CERTAIN PLANT VIRUS INCLUSIONS

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ABSTRACT.—The trypan blue method for detecting certain virus crystallin inclusions is greatly facilitated by a pre-stain treatment with a diethyl-ether-ethyl-alcohol solution made up in physiological saline (5 ml. of diethyl ether and 10 ml. of ethyl alcohol diluted to 100 ml. with physiological saline solution). Maceration of the study material in trypan blue solution also gives good results.

INTRODUCTION

The detection of the presence of certain virus inclusions in host plants became a relatively simple laboratory procedure when McWhorter (1941 a & b) discovered that Phaseolus virus 2 and various lily viruses produced crystallin inclusions which could be stained with an aqueous solution of trypan blue. Subsequent studies of the inclusions of Phaseolus virus 2 resulted in the perfection of more rapid modifications of the original technic.

TECHNICS

The tissue studied, in the majority of cases, was integumentary, consisting of epidermal peelings from diseased and comparable healthy leaves. When these strippings were placed in 0.05% trypan blue in physiological saline solution (1941b), with no pre-stain treatment, 30 minutes to 3 hours were required for the staining reaction to reach its end-point, i.e., a brilliant blue coloration of nuclei. The length of staining time seemed to correlate directly with the age of the plant tissue. Trials showed that crushed cells stained readily, indicating that the foliar cells became less permeable to trypan blue as the plants aged. The problem of increasing permeability was solved by a two-minute pre-stain treatment of the tissue with an aqueous solution of 5% diethyl ether and 10% ethyl alcohol. This solution is made as follows: To 50 ml. physiological saline solution add 10 ml. of 95% ethyl alcohol. To this mixture add 5 ml. of diethyl ether and stir until solution is complete. Make up to 100 ml. with physiological saline solution. If excessive plasmolysis occurs, decrease ether content to 3% and increase alcohol content to 15%.

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The use of this solution resulted in a standard, dependable technic which reached a stain reaction end-point within 3 to 4 minutes after obtaining the leaf strippings. The two-minute treatment in ether-alcohol solution caused very little plasmolysis. Staining in trypan blue without previous fixation resulted in varying degrees of nuclear swelling.

Another method, referred to as the *crush* technic, was used for plants from which it was difficult to obtain leaf strippings. A small piece of leaf or stem is placed in a drop or two of 0.05% trypan blue on a slide and then rubbed until macerated with a specially prepared glass spatula having a flat ground glass surface. The preparation is then ready for immediate observation since the remaining stain serves as a corrective white light filter. The nuclei swell, but the size and shape of the crystallin inclusions are unaffected. Very often the nuclei may be observed floating in the mounting medium with clearly visible, well-stained crystals contained in their nucleoli. Less often, viroplasts have been observed floating free or attached to nuclei.

The drastic crush method is of diagnostic value only when the cell inclusions are very easily recognized. Using this technic for comparative purposes on viroous tissue not previously studied by other methods would only result in confusion and errors. For checking the presence of the crystals produced by Phaseolus virus 2, however, the crush technic was efficient since the cytological evidences of this virus are unusually stable. This method is very rapid, allowing free mingling of cell contents with trypan blue in a manner involving no permeability problems.

Simple free-hand sections were made to observe cytological virus evidence in petioles and stems. The parenchyma cells of stem sections proved very satisfactory for observation, but the attached trichomes were of no value in the case of these leguminous hosts.

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A MODIFIED TECHNIC FOR SALIVARY-GLAND CHROMOSOMES

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ABSTRACT.—This technic was worked out for making permanent mounts of salivary-gland squashes of strains and species of *Drosophilidae*, which yield material difficult to work with. It includes a prior fixation in a modified Carnoy's fluid, staining in solutions of orcein in 60% or 70% acetic acid, part dehydration in the vapor and liquid of a 95% solution of alcohol, and direct mounting in Euparal by means of the drainage-method.

During a study of the salivary-gland chromosomes of various strains of *Drosophila melanogaster* and other species of local *Drosophilidae*, the usual aceto-carminic technic of fixation staining, as recommended by Painter (1934), was followed, using an iron-aceto-carminic prepared according to the method of Belling (1926). While this technic yielded satisfactory results in the case of larvae which were fed on yeast-rich cultures at low temperatures, very variable and unsatisfactory results were obtained in the case of larvae from weak and slightly over-populated stocks, when the occasion arose to examine such, and also in the case of various species of the genus *Zaprionus*.

These variable results were ascribed to the staining capacity of the available batches of dye, and aceto-orcein was then tried out. For this dye also definite differences were found to exist in staining capacity in the case of the above-mentioned difficult stocks; ultimately the two best batches of the orcein available were selected, and these were, firstly, a British Drug Houses product obtained about fifteen years ago, and secondly, a product of Grüber obtained just prior to the war.

The various difficulties occurring with the larvae of certain weak strains and of the various local species of *Zaprionus* are: (1) The salivary-gland chromosomes do not stain very easily, or differentially, and (2) the chromosomes do not spread evenly during the squashing of the glands; they are soft and fragile, or tightly coiled.

The first difficulty has also been noted by Warters (1944), who modified the technic for certain groups showing varying affinities for the stain by a variation in the time used for staining and destaining. Both difficulties are also mentioned by Kodani (1939), who

ascribed the lack of proper staining capacity and the inability to render chromosomes flexible enough so they will spread out on crushing as due to a loss of fixing power of the acetic acid after being boiled with the dye; he advised a prior fixation with acetic acid before staining. A prior, or additional fixation, has also been recommended by various workers for the purpose of reducing the staining of the cytoplasm and thus increasing differential staining, and also for the hardening of the chromosomes: acetic acid for mitotic chromosomes by McClintock (1929), Carnoy's fluid for mitotic chromosomes by Koller (1940), and chloroform for the salivary-gland chromosomes of *Sciara* by La Cour (1941).

A prior fixation with Carnoy's fluid was utilized and found to improve the spreading of the chromosomes, to decrease the time of staining and increase the selectivity of the stain. The following technic for fixing, staining and spreading, and mounting salivary glands of difficult strains was then evolved. The method has provided uniformly good slides of salivary-gland chromosomes, and may be divided into its various procedures.

1. *Prior Fixation.* A preliminary fixation is given in a modified Carnoy's fluid (12 ml. absolute alcohol, 6 ml. chloroform, 1 ml. glacial acetic acid) as recommended by Koller (1940).

The glands were dissected out in Ringer and transferred on a flat-bladed dissecting needle to a drop of the fixing fluid on a well-slide. Up to certain limits the period of fixation varies; the longer the glands remain in the fluid, the greater is the extent of spreading attained during the crushing of the glands. The optimum time for the B.D.H. stain was determined as $\frac{3}{4}$ -1 minute, and for the Grüber stain $1\frac{1}{2}$ -2 minutes.

2. *Staining and Spreading.* The stains found to give the most satisfactory results were the following: The B.D.H. and Grüber stains as 2% solutions in 70% acetic acid, made up by the method of La Cour (1941), and the Grüber stain in 60% acetic acid, made up and diluted as recommended by Demerec and Kaufmann (1945).

The glands are transferred from the fixing fluid to a few drops of the stain on a well-slide; the optimum period of staining was found to be 7-10 minutes for the B.D.H. stain and 5-7 minutes for the Grüber stains. It must, however, be noted that the time of staining varies for the different strains and species. The glands are then removed by means of a pipette to a fresh drop of stain on a clean slide and the cover glass is applied, the excess stain drained off, and a square of blotting paper laid over the cover glass.

The spreading of the chromosomes is affected by the amount of

pressure applied on the cover glass. Various workers use various methods, such as the ball of thumb, tapping with a blunt needle, or rolling with a glass rod. The following method has, however, been found to be the most satisfactory for the even spreading of the chromosomes, very often in the form of parallel arms: The square of blotting paper is lightly pressed to flatten the glands and remove excess stain, and a glass rod is then rolled firmly once across the paper. The amount of pressure exerted when rolling may be varied, so that varying degrees of spreading and stretching of the chromosomes may be attained.

3. *Mounting.* After trying out the method of McClintock (1929) for making aceto-carmine smears permanent, as well as the modified method recommended by Darlington and La Cour (1942), it was found that more simplified methods gave satisfactory results. For permanent mounts it seems advisable to spread, distribute evenly and dry a thin film of albumin on the slide, as described by Demerec and Kaufmann (1945).

Two methods of dehydration and mounting are utilized:

(a) The routine method is that recommended by Demerec and Kaufmann (1945). The slide is placed in a covered dish containing 95% alcohol so that the lower edge of the cover glass dips in the alcohol. After 6–12 hours the slide is immersed in 95% alcohol for 1–2 hours. The cover glass is then gently pried free from the slide while still in the alcohol, by inserting a sharp-pointed instrument beneath one edge, the excess alcohol is quickly drained off, a drop of Euparal is placed on the part of the slide containing the salivary glands, and the cover glass is applied.

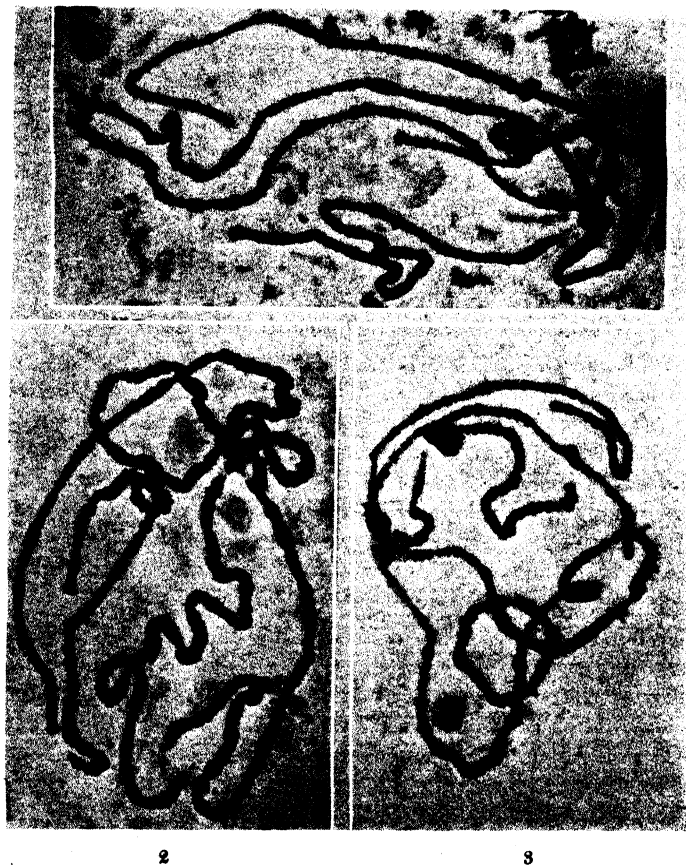
(b) Where especially fine and well-spread preparations are obtained, any accidental loss of chromosomes, due to disruption while lifting the cover glass, may be obviated by using the second method of dehydration and mounting. A modified version of the vapor method of Bridges (1937) for changing reagents is used. The slide is laid on a metal rack above the surface of 95% alcohol in a tightly covered dish. The edges of the cover glass are moistened with a drop of 70% alcohol and the slide is left for 24–48 hours in this dish with a differential vapor pressure.

After a displacement of liquids has occurred through evaporation and diffusion, the slide is immersed in 95% alcohol for 6–12 hours. It is then again placed on a metal rack above the surface of 95% alcohol in a covered dish, a square of blotting paper is placed along one edge of the cover glass and a drop of Euparal is applied along the opposite edge; the alcohol vapor in the atmosphere of the covered dish keeps

the Euparal in a liquid state, and by means of this drainage method the alcohol under the cover is replaced by Euparal.

This method has produced fine permanent preparations, however only if the Euparal rapidly and totally displaces the alcohol, since otherwise a fading takes place of the stain in the bands.

1



Salivary gland chromosomes stained by technic described. Fig. 1 (top), 2 (left) and 3 (right) as described in text.

Fig. 1-3 show nuclei of larvae from ordinary, slightly over-populated, laboratory stocks of *D. melanogaster*, i.e. without being prepared by means of special feeding and temperatures. The photomicrographs were taken through a Zeiss apochromat 20 \times , n.a. .65

and a compensating eyepiece 15 \times ; the final magnification was $\times 450$. Exposure was 2 seconds on Kodak Contrast Process Ortho, while the prints were D76 for 10 minutes. Fig. 1 and 2 are of nuclei stained with the B.D.H. orcein and mounted in Euparal by the drainage-method. Fig. 3 is of a nucleus stained with the Gr bler stain and mounted by the routine method.

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BRILLIANT CRESYL BLUE AS A STAIN FOR CHROMOSOME SMEAR PREPARATIONS

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ABSTRACT.—Brilliant cresyl blue is substituted for carmine in the acetocarmine technic. A mixture of one part of 1% toluidine blue and three parts 0.75% brilliant cresyl blue dissolved in 45% acetic acid gives good results. Propionic acid may be used instead of acetic acid for more rapid penetration.

After several unsuccessful attempts to stain the salivary-gland chromosomes of *Drosophila macrospina macrospina* with acetocarmine, brilliant cresyl blue (a basic stain) was substituted for carmine and the chromatic discs were stained a dark blue. Brilliant cresyl blue is the stain commonly employed to show platelets and reticulated erythrocytes (Robertson, 1917). In the substitution for carmine in the acetocarmine, the brilliant cresyl blue is dissolved in 45% acetic acid and filtered. It is unnecessary to boil the stain.

The stain technic is as follows:

- (1) Dissect out the salivary glands of the *Drosophila* larva in the usual manner (Painter, 1934).
- (2) Place the glands in a small glass vial and add 5 drops of 0.75% brilliant cresyl blue¹ in 45% acetic acid.
- (3) Stain for 30 to 45 minutes.
- (4) Pour the contents of the vial onto a clean slide.
- (5) Tilt slide to expose glands and blot off surrounding liquid.
- (6) Break nuclei by tapping or pressing the cover slip.
- (7) If overstained, introduce a drop of 45% acetic acid under the cover slip.
- (8) Ring the cover slip with solution of Clarite dissolved in xylene.

In some cases good results were obtained if the glands were first fixed in 45% acetic acid and then placed in the stain. In several strains of *Drosophila macrospina macrospina* the salivary-gland chromosomes would stain if one part of 1% toluidine blue² were added to three parts of brilliant cresyl blue. It was also found that in some strains the salivary-gland chromosomes would be somewhat resistant to brilliant cresyl blue in acetic acid, but would stain more

¹The lot used bore the certification No. NV-17.

²The lot used bore the certification No. NU-2.

readily if propionic acid were used instead of acetic acid. The Clarite-xylene ring dries fast and leaves an air-tight seal. Temporary slides will keep at least six months.

The advantages of this staining method are: (1) If salivary chromosomes resist the acetocarmine stain, the mixture of brilliant cresyl blue with acetic or propionic acid mixture may stain the chromatic bands. (2) There is no "luck" required to make a good staining solution as in acetocarmine (Smith, 1947). (3) Blue bands on a gray background are more distinct than those obtained by using acetocarmine.

The disadvantages are: (1) The mixture of acetic acid and brilliant cresyl blue is ink-like in appearance, and it is difficult to orient the gland in the stain. (2) A larger volume of stain is used, and hence the need of glass vials. There is a chance that loss of a gland may occur in transfer from vial to slide.

It appears that other basic dyes dissolved in acetic acid may be used in the rapid smear method to demonstrate chromosomes. Propionic acid may be used instead of acetic acid to facilitate penetration. Thus the research worker need not depend upon carmine or orcein, for he has a larger selection to help solve his staining problem.

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AN ACETO-CARMINE SQUASH TECHNIC FOR MATURE EMBRYO SACS

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ABSTRACT.—A method is described by which whole embryo sacs of *Nicotiana*, *Petunia* and no doubt of certain other genera can be obtained readily in aceto-carmine ovule squashes. Although application of the technic to megagametogenesis and fertilization stages is stressed in this paper, use of the method allows development to be traced from the archesporium up to the second or third division of endosperm nuclei. The success of the technic depends on four phases:—1) fixation in a medium that causes cell and nuclear structures to become pliable, yet rigid enough that their spatial relationships are not greatly distorted in squashing; 2) heat, which apparently increases the cohesion of cytoplasmic and nuclear constituents; 3) maceration to separate the embryo sac from surrounding cells; and 4) the use of a stain that differentiates the various nuclear structures as well as those of the cytoplasm. Staining of the cytoplasm, essential in some embryological investigations, is one advantage of the aceto-carmine squash method over the Feulgen procedure. In contrast to the Feulgen ovule squash method the aceto-carmine technic will probably be most useful in genera having numerous small ovules. Advantages and defects of the aceto-carmine procedure as compared with the paraffin technic are discussed, likewise the possible usefulness of the former in studies of sterility and in certain other special connections.

Ovule squash technics based on the Feulgen reaction (Hillary, 1940) or combined with acetic-lacmoid (Darlington and La Cour, 1942) are known. Both methods are applicable chiefly to genera with large ovules, due to the difficulty of handling small ovules removed from the placenta. In the aceto-carmine technic, however, ovules are not separated from the placenta until ready to be squashed, and therefore the procedure is as adaptable to small as to large ovules. Actually small size is advantageous, since numerous minute ovules can be included in a single preparation. Although embryo-sac size has not been tested as a factor in success of the aceto-carmine technic, a certain maximum size may perhaps limit application of the method, as in the case of the Feulgen procedure (Hillary, 1940).

Since resting nuclei of the embryo sac stain only faintly with basic

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fuchsin, the Feulgen method is valuable chiefly in studies of nuclear divisions. This disadvantage, plus the failure of cytoplasm to stain, causes the Feulgen technic, at least when used without counter-staining, to be relatively useless in many embryological investigations. With aceto-carmin, following the proper pre-treatment, these difficulties are not encountered. Without counter-staining, chromatin in all phases is well stained and differentiated from the cytoplasm, which is likewise stained to some extent; the positions of vacuoles and cytoplasmic strands can be made visible; and in fertilization stages the pollen tube cytoplasm is readily distinguished from that of the embryo sac.

This paper emphasizes application of the aceto-carmin method to stages from the 2-nucleate embryo sac through fertilization (i.e. stages impossible to find intact in aceto-carmin squashes unless special pre-treatment such as that described below is used). However, archesporia, meiotic stages and changes occurring in the four megasporia can be detected in ovule squashes of *Nicotiana*. Thus, in this genus at least, the entire sequence from the archesporium through megasporogenesis, megagametogenesis, fertilization and the first few divisions of endosperm nuclei can be followed. In genera in which the free-nuclear type of endosperm formation occurs, development might be traced even further.

PROCEDURE

Since this technic may be useful to embryologists who are not familiar with squash methods in general, the procedure will be discussed in considerable detail.

Fixation. The fixative is 4 parts chloroform, 3 parts absolute ethyl alcohol and 1 part glacial acetic acid. Evacuation of air from ovaries immersed in the fixing fluid is not necessary. To the high chloroform content of the fixative is attributed the pliability of cell structures essential in keeping embryo sacs intact through the subsequent treatment. After the standard fixatives used in connection with aceto-carmin staining—alcohol-acetic (3:1) or alcohol-chloroform-acetic (6:3:1)—embryo sacs are so brittle that they are easily fragmented. In addition to flexibility, fixation of nuclear components superior to that of either 3:1 or 6:3:1 is also provided by the fixative.

The optimum fixation period extends from two days to three weeks. With less than 44–48 hours in the fixing fluid, embryo sac nuclear and cell membranes are not toughened enough to resist pressure applied in squashing. After approximately three weeks in the fixative, although

whole embryo sacs are obtained, the differentiation of nuclei and cytoplasm begins to deteriorate and chromosome structure is less sharply defined. Deterioration is slow, however, and material fixed in this formula may not be completely useless until more than a year has passed. (Fig. 1 to 4 were photographed from ovules fixed 3 months before being squashed; Fig. 5 to 10 from material squashed after 2 days to 3 weeks in the fixative.) Evidence obtained recently indicates that addition of chloroform to the vial of ovaries two or three days after fixation is begun, especially advisable if evaporation of the fixative has occurred, slows the deterioration process. Unlike tissues fixed in 3:1 or 6:3:1, those fixed in the formula given above should *not* be transferred to 70% alcohol for storage, since alcohol causes the cytoplasm to darken and definition of nuclear structures to become inferior.

Heat and mordanting treatment. Several ovaries are removed from the fixing solution, drained on absorbent paper, and placed in a vial of 4% aqueous solution of iron alum. This vial and two vials of distilled water are placed in a water bath which is then heated rather rapidly to 75°C. The water bath is kept at that temperature for the following 7 minutes. After 3 minutes at 75°, ovaries are transferred to one of the vials of water; after 2 minutes they are changed to the other vial of water, in which they remain for another 2 minutes. The vial of ovaries is then removed from the bath and cold water is added to its contents to lower the temperature. After 2 or 3 minutes in cold water the ovaries are ready to be macerated. In the case of large ovules the hot iron alum and hot water treatments should perhaps be prolonged beyond the 7-minute total. Embryo sacs of some genera may respond more favorably to heat applied at a lower temperature (for example, 65°) and for a longer period of time (up to 1 hour in hot iron alum and an equal period of time in hot water).

The heat treatment apparently binds embryo sac constituents together, enabling them to cohere through subsequent treatment. In addition to mordanting the stain, the iron alum protects nuclei from the action of hot water, as indicated by the fact that substitution of a 7-minute hot water treatment in place of the 7-minute heat treatment described above caused shrinkage of nuclear membranes and hydrolysis of chromatin. Other iron compounds might be more effective than iron alum. Solutions of iron acetate were found to be superior to iron alum for nuclear differentiation but inferior in preserving embryo sacs intact, probably because acetic acid was used as the solvent since iron acetate is practically insoluble in water. How-

ever, addition of 10 drops of a saturated solution of iron acetate in 45% acetic acid to 10 ml. of 4% aqueous solution of iron alum improves chromatin definition without detracting materially from embryo sac integrity. Results of controlled experiments showed that the improvement in definition was due to the iron acetate, not to the acetic acid in which it was dissolved.

Maceration. Ovaries are macerated in 50% hydrochloric acid for 10 minutes. Maceration of large ovules might be improved by longer immersion in the acid. Then they are washed in several changes of distilled water for a total period of at least 20 minutes, after which ovules are ready to be squashed. Differentiation of nuclei and cytoplasm usually is not harmed by soaking in water for several hours, and maceration is improved by it. However, the longer the tissues remain in water, the softer the embryo sacs become and the more delicate the squashing must be in order not to destroy their continuity. *Nicotiana* and *Petunia* ovules can be squashed successfully even after 2 hours in water.

Squashing and staining. An ovary is removed from the water and is drained on absorbent paper. While it drains, one or two ordinary dissecting needles from which the glaze has been removed are soaked in a drop of iron-aceto-carmine² on a slide, in order to add more iron to the stain; when the aceto-carmine begins to assume a slight purplish cast, enough iron has been added. (An abundance of iron is required to stain embryo sac nuclei immediately.) The ovary is placed in the drop of stain, the ovary wall removed with dissecting needles and ovules are scraped from the placenta into the stain. Fifty to one hundred ovules of the size found in *Nicotiana* species can be included in a single preparation to be covered by a 22×22 mm. cover slip. (Additional ovules may still adhere to the placentae and in case they are to be used for another preparation, the ovary may be transferred from the drop of stain to another slide; the ovules are sufficiently moist that they will not dry out for some time.) Bits of tissue other than ovules are removed from the stain. With a flat-bladed smearing implement³ not constructed of iron, the ovules are

²Iron-aceto-carmine is prepared by refluxing for 4 hours 45% acetic acid with carmine crystals added in excess of the amount readily soluble in the quantity of acetic acid used. After refluxing, the stain is rapidly cooled, then filtered. To a small quantity (ca. 25 ml.) of the stock aceto-carmine thus prepared a saturated solution of iron acetate in 45% acetic acid is added; the amount of iron acetate that can be added without causing precipitation varies with the lot of stain, but is usually between 5 and 10 drops to 25 ml. of aceto-carmine.

³A toothbrush handle, with the end cut diagonally between upper and lower surfaces and the diagonal face polished, makes a useful squashing instrument.

tapped gently, never with a smearing motion, until the cells are well separated. The material is then distributed as evenly as possible over the area to be covered by the cover slip. As few motions as possible should be involved in separating or in spreading the cells; the less contact the embryo sacs have with the squashing instrument the more intact and the less distorted they will be. A cover slip is applied and the slide is heated over an alcohol flame, but the stain must not be allowed to boil. (Moderate heat swells cells and nuclei, improves spacing of nuclear components, intensifies staining of nuclei and decreases it in the cytoplasm.) The preparation is then examined⁴ to determine whether the desired stages are represented, the stain sufficiently intense, and whether the embryo sacs require flattening. To intensify the stain the slide is alternately heated and cooled several times. To flatten and to spread the sacs, the slide is heated and while still hot is inserted between the two halves of a folded paper towel and pressure is applied to the cover slip region with the finger tips; lateral movement of the cover slip should be avoided. For study of nuclear structure and chromosome number, pressure is often desirable, although it may distort or blur outlines of embryo sac cells and positions of vacuoles.

The preparation may be sealed with a mixture of half gum mastic and half paraffin, applied to cover slip edges with a hot metal applicator. If permanent preparations are desired, any of several procedures may be used (Bridges, 1937; Hillary, 1938; La Cour, 1947; McClintock, 1929). A modification of Bridges' vapor method is being published by the author and has the advantage that the cover glass is not removed, thus possibility of loss or overlapping of desirable material in the finished preparation is eliminated (Bradley, 1948).

Destaining. Frequently the best preparations are temporary ones that have been overstained and are later destained. Destaining must be done with caution, however, since material usually cannot be restained after it has been destained. If material has been overstained initially or has become too dark with age, the sealing mixture is scraped away from two opposite edges of the cover slip and a mixture of *ca.* 5 parts 50% acetic acid and 1 part aceto-carmin is then drawn through the preparation by placing a drop of the mixture in contact with one open edge and absorbent paper in contact with the other. When the color of the stain taken up by the paper begins to appear pink or red, rather than purplish, no more liquid should be drawn through, even though cells in the preparation are still too dark,

⁴In any microscope studies of aceto-carmin preparations, a blue-green light filter is invaluable.

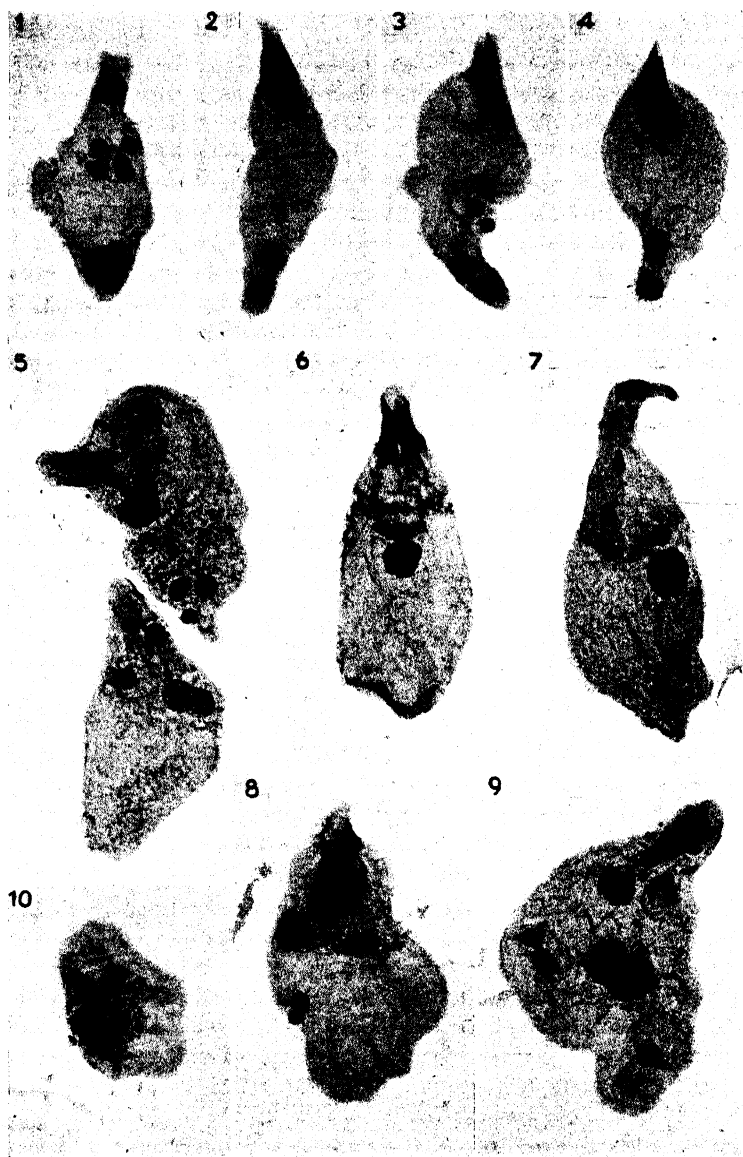


PLATE 1

All photomicrographs are of embryo sacs of *Nicotiana tabacum* and were selected more for nuclear phase or structure or for fertilization stage than for cytoplasmic features. The micropylar end is uppermost in all sacs. Somatic cells around the embryo sacs were blocked out, but nothing within the sac boundaries was retouched. All figures $\times 275$. Description of figures on p. 35.

1. Prophase of the second division. Note the dense cytoplasm around the nuclei and the large vacuole between them, part of which is overlaid by somatic cells.

2. Prophase of the third division. Some of the lines in the central region are due to crumpling of the sac wall. The same applies to Fig. 1, 3 and 4.

3. Metaphase of the third division. The sac was badly distorted in squashing, and somatic cells overlap part of it. The end regions have not been damaged and show the different planes of division.

4. Anaphase of the third division. The two chromosome groups in each of the terminal division figures are partially or totally superimposed due to the planes of division. Note the greater cytoplasmic density and more pointed outline of the micropylar end, which appear also in Fig. 2 and 3.

5. Two mature embryo sacs. Cell outlines are blurred due to pressure on the cover slip. In the more distorted, upper embryo sac the three antipodals are seen, also the fusion nucleus, egg and synergid nuclei; the egg cell outline is vaguely indicated. In the lower sac no antipodals are present (see text); a ring of relatively dense cytoplasm is seen around the egg nucleus; the polar nuclei appear to be fusing; synergid cells are distorted but the outline of the one to the right is dimly represented; two darker lines along the synergid apices are remnants of the refringent outer wall of the megaspores (see text) and are seen also in Fig. 2 and 6.

6. A mature embryo sac. The large central nucleus is probably not the fusion nucleus but rather the single polar nucleus formed in this sac, judging by the size of its nucleolus (cf. Fig. 5 and 7) and by the types of abnormalities found in this species. If that is the case, the first division failed, which also accounts for absence of antipodals. The outline of the egg cell up to the dense cytoplasmic region of the synergids may be traced, and with less certainty the basal outlines of synergid cells, slightly above the egg nucleus and superposed by the egg cell. Internal nuclear structure of the synergids is absent because the nuclei are becoming pycnotic. Positions of starch grains are indicated by pale circular areas surrounded by darker lines of cytoplasm and are seen also in Fig. 9.

7. Pre-fertilization. The pollen tube has entered the embryo sac and the sperms are seen as two small curved nuclei at the base of the pollen tube cytoplasm. The nucleus uppermost in the sac, apparently lying in the cytoplasm of the pollen tube, is the degenerating synergid nucleus. (The nucleus of the other synergid is missing, probably due to the squashing.) Between the synergid nucleus and the sperms is the degenerating vegetative nucleus of the pollen tube. To the right of the sperms is the egg nucleus, somewhat out of focus, and below that is the large fusion nucleus. Near the base of the sac is the single antipodal.

8. Pre-fertilization, somewhat more advanced than in Fig. 7. At the lower left, overlapping the sac, are two somatic nuclei. Above is the single polar nucleus, and the small elongated nucleus to the right of it is a sperm. The other sperm nucleus, slightly curved, is to the left of the egg nucleus and appears to have entered the egg cell. The irregularly shaped chromatin body to the upper right of the polar nucleus is the degenerating vegetative nucleus. Appearing to lie in the extremely dark pollen tube cytoplasm, but actually superposed by it, is the nucleus of the undamaged synergid. To the upper right of that is the pycnotic nucleus of the synergid ruptured by the pollen tube.

9. Fertilization—an advanced stage. The uppermost chromatin mass, near the base of the darkly stained pollen tube cytoplasm, is the degenerating synergid nucleus. Just below it is the pycnotic vegetative nucleus, and below that a "nucleus" that is actually the fusing egg and sperm nuclei, surrounded by the deeply stained cytoplasmic structures characteristic of late fertilization and zygote stages. The larger nucleolus, contributed by the egg, is left of center of the fusing nuclei and the dim outline of the sperm appears toward the right. The intact synergid is to the right of the vegetative nucleus; the lower part of its cell outline is visible. Apparently the second sperm has merged with one of the polar nuclei prior to their complete fusion. To the left of the polar nuclei are two antipodals that have migrated from the chalazal end of the sac, a common occurrence in this species. The third antipodal is seen below the polar nuclei.

10. Metaphase of one of the early endosperm nuclear divisions. Although some of the chromosomes are not in good focus, all 72 can be counted. The haploid number of *N. tabacum* is 24.

since the excess acid will continue to destain for a time. Any liquid left beside the cover slip should be blotted up before fresh sealing mixture is applied to *all* edges of the cover slip.

SUMMARY OF PROCEDURE

- (1) Fix ovaries for at least 2 days in 4 parts chloroform: 3 parts absolute alcohol: 1 part glacial acetic acid.
- (2) Drain ovaries and place in a vial of 4% aqueous iron alum.
- (3) Place vial and 2 vials of distilled water in a water bath, heat bath to 75°C., and keep at that temperature for 7 minutes.
- (4) Leave ovaries in iron alum at 75° for 3 minutes.
- (5) Transfer to vial of water—2 minutes at 75°.
- (6) Change to other vial of water—2 minutes at 75°.
- (7) Remove vial of ovaries from the bath and pour cold water into the vial. Leave ovaries in cold water for 2–3 minutes.
- (8) Macerate in 50% hydrochloric acid—10 minutes.
- (9) Wash in several changes of distilled water—at least 20 minutes in water, altogether.
- (10) Drain an ovary and place in a drop of iron-aceto-carmin on a slide. Scrape ovules from the placentae into the stain. Remove bits of tissue other than ovules from the stain.
- (11) Tap the ovules gently with a flat-bladed squashing implement until the cells are separated.
- (12) Apply a cover slip and heat the slide over an alcohol flame, without boiling the stain.
- (13) Examine the preparation to see whether pressure and/or more heating is required.
- (14) When the preparation is adequately stained, seal it or make it permanent.

DISCUSSION

Although many more somatic cells than embryo sacs are present in ovule squashes, a considerable number of embryo sacs in good condition are to be found in a successful preparation. Some sacs are unavoidably distorted, broken or covered by somatic cells, but an average squash may contain much more usable material than an average paraffin section slide; [2- and 4-nucleate sacs are most susceptible to crumpling of cell walls during the squash procedure, because of their large central vacuoles (cf. Fig. 1–4 with 5–9)]; 2- to 8-nucleate sacs are easily recognized by their size and the characteristic appearance of their nuclei. Earlier stages, although not as readily distinguished from somatic cells on the basis of cell size, can be identified by cell shape and the staining quality and size of their nuclei.

Compared with the paraffin technic, the aceto-carmin squash method for embryo sacs has certain advantages and also some disadvantages. Among the former are: 1) great reduction in time and labor involved; 2) superiority in showing (a) nuclear differentiation, and number and morphology of chromosomes and (b) changes in

sperm and "x-body" structure from time of penetration of the pollen tube into the ovule to post-fertilization stages (cf. Goodspeed, 1947); 3) absence of the uncertainty of interpretation encountered in studies of paraffin sections when the embryo sac must be reconstructed from two or more sections; and 4) adaptability of squashed embryo sacs to photomicrography (Plate 1). On the other hand, the development of nucellus, integuments, etc. in relation to embryo sac development cannot be followed in squash preparations. Also, orientation of megaspores and embryo sacs in relation to chalazal and micropylar ends of the ovule may be lost in some genera. In *Nicotiana* and *Petunia*, however, orientation is possible even in mature sacs, due to retention at their synergid apices of traces of a peculiar refringent wall that had earlier surrounded meiotic and megaspore cell groups. (Fig. 2, 5 and 6). A third defect of the squash technic would apply only to genera in which the various embryo sac cells are separated by walls that include membranes subject to action of the macerating fluid, in which case the cells would be separated.

Some types of abnormal embryo sac development can be detected with much greater certainty in squash preparations than in paraffin sections. For example, in *Nicotiana tabacum* different mature embryo sacs from the same ovary showed variation in number of antipodals; some had three, others one, and in still others none was present (Fig. 5 to 9). Studies of earlier stages showed that failure of one or two nuclear divisions was the cause. In paraffin sections such differences in number of antipodals might have been interpreted as loss of one or more nuclei in sectioning. Other abnormalities seen in squash preparations included embryo sacs with supernumerary nuclei, frequently due to extra divisions of antipodals and in some cases to divisions of other nuclei. Several instances of twin embryo sacs were seen, probably of the type developing within a single nucellus, since the two sacs remained attached through the squashing process. In addition, a few cases of multiple pollen tubes in embryo sacs were found in *Nicotiana longiflora* (Goodspeed, 1947).

In genera in which the aceto-carmin squash method is successful, studies of the cytology of sterility may be facilitated by its use. Pycnosis of nuclei should be a clue to the stage of megasporogenesis, megagametogenesis or early post-fertilization beyond which development does not proceed. Rather early stages of nuclear degeneration are identified more readily in aceto-carmin squashes than in paraffin sections stained with hematoxylin. (Examples of degenerating nuclei, not connected with sterility, however, are seen in Fig. 6 to 9. Also cf. illustrations, Goodspeed, 1947.) Another type of sterility, not due to nuclear and cell degeneration, has been detected in ovule squashes of several *Nicotiana* species in which no seed was setting early in the flowering season—a common phenomenon in many genera. In ovaries fixed at least 48 hours after anthesis, fully differentiated 8-nucleate embryo sacs were rare and in many only 2- and 4-nucleate sacs were seen. In most preparations no pollen tubes were found; in a few, the occasional tube seen had not penetrated an embryo sac. Lack of synchronization in floral development is no doubt a factor in sterility here—perhaps the only factor. Unfor-

tunately ability of pollen to germinate was not investigated, but since immaturity of ovules probably implies a similar state in stigma and style, it may be assumed that stigma conditions were not appropriate for pollen germination.⁵

Through use of the aceto-carmin squash method certain problems long discussed by embryologists can be solved or at least brought nearer solution. One is the nature of "x-bodies." From observations of fertilization stages in ovule squashes and of vegetative nucleus phases in stigma, pollen tube⁵ and ovule squashes, no doubt remains that in *Nicotiana* and *Petunia* one of the "x-bodies" is the degenerating vegetative nucleus and the other the degenerating nucleus of the synergid damaged by penetration of the pollen tube into the sac (Fig. 7, 8 and 9. Cf. also Goodspeed, 1947). In aceto-carmin squashes there is no possibility of confusing either of these two degenerating nuclei with cytoplasmic structures. Although the Feulgen stain is more specific for chromatin, staining only structures containing desoxyribose-nucleic acid when skillfully applied, aceto-carmin is almost as useful in differentiating nuclei (either healthy or pycnotic) from cytoplasmic constituents. Only in certain cases (tannins, or contents of abnormal cells or pollen tubes) are cytoplasmic granules stained by aceto-carmin with the same intensity as the nucleic-acid-containing chromatin and nucleoli. Usually, deeply stained cytoplasmic particles can be distinguished from nuclear elements by differences in refringence, and often the former can be made to disappear from view by opening wide the sub-stage diaphragm, while the latter remain visible.

Certain evidence in regard to cellular structure of embryo sac components is obtainable from squash preparations. In *Nicotiana* and *Petunia* the outlines of egg, synergid and antipodal cells and also the disposition of cytoplasmic regions of different densities within the cells are frequently clear (Fig. 6, outline of egg cell. Cf. also illustrations, Goodspeed, 1947). In some cases definite membranes are apparent, separating the cells from one another and from the central region containing the polar nuclei or fusion nucleus. That the membranes are not subject to hydrochloric acid action, (at least to a visually detectable degree) and that the cells are not held together merely by the wall surrounding the entire sac is indicated by

⁵In this connection, a simple method of preparing material for investigation of pollen germination and pollen tube development *under natural conditions* (including growth rate, generative nucleus division, sperm and vegetative nuclear structure, etc.) involves only the following few steps: fixation of stigmas and styles in the formula given for ovule squashes, maceration of the stigma in hydrochloric acid (50% for 10 minutes) before squashing it in iron-aceto-carmin; scraping out of pollen tubes from the interior of successive regions of style that have been slit lengthwise and the cut edges folded back, teasing apart of pollen tubes in a drop of iron-aceto-carmin; adding a cover glass, heating the slide, whether it holds pollen tubes from the style or macerated stigma cells and pollen grains, and applying pressure to the cover glass if necessary. To stain the vegetative nucleus a large amount of iron in the stain may be required, likewise several alternations of heating and cooling.

the manner in which embryo sacs are disrupted by extreme pressure. Breakage is almost invariably in the central region, and remnants of the sac include parts of the central "cell" plus either the intact egg apparatus or the group of antipodals, neither of which shows any tendency toward separation of its constituent cells, even though the pressure applied was sufficient to spread them considerably. Failure of the membranes to be weakened by hydrochloric maceration suggests that they may be similar in structure to membranes between recently formed somatic sister cells. Such sister cells usually remain together as a unit after maceration, although the membrane separating them must have been developing during the time required for their nuclei to progress from anaphase-telophase to a moderately advanced resting stage. (Sister cells can be recognized by their identical chromatin state, cell and nuclear size and by the mirror-image positions of their nucleoli.) Incomplete formation of the membranes—incomplete either in chemical changes leading to formation of pectic substances subject to hydrochloric action, or in the continuity of such pectin molecules—is probably responsible for failure of somatic sister cells to separate after maceration, and the same may be true in regard to embryo sac cells.

Although the aceto-carmin method has been tested chiefly on *Nicotiana* species and one *Petunia* species, all of which have given satisfactory results, it was tried also on two or three ovaries of one species of each of the following genera: *Scilla*, *Aloe*, *Aeonium*, *Papaver*, and *Aquilegia*. In the case of the two monocots, *Scilla* and *Aloe*, whole embryo sacs were not obtained, because a group of cells at the chalazal end of the embryo sac could not be macerated with hydrochloric acid and attempts to separate it from the sac resulted in rupturing of the sac. In *Aeonium* the method was partially successful and with changes in heat or maceration schedules would probably yield satisfactory results; the embryo sac of *Aeonium* is contained in a thick wall which ruptures in some cases to allow cytoplasm and nuclei to flow out intact. In *Aquilegia* and *Papaver* preparations complete 8-nucleate sacs were found, and only minor variation, if any, of the method should be necessary. From these investigations it may be predicted that the technic described here will be most successful in genera having many small ovules, more commonly occurring in some dicot families than in those of monocots. The obstacles to maceration presented in *Scilla* and *Aloe* will perhaps be encountered also in genera related to them. However, a macerating agent might be found that would permit the aceto-carmin squash procedure to be adapted to such genera. Probably in the Solanaceae in general, and perhaps in related families as well, no modification of the technic will be required.

It is hoped that other investigators will develop variations of the aceto-carmin squash method that will extend its use to studies of embryo sac development in genera that will not respond favorably to the method given here.

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A METHOD FOR MAKING ACETO-CARMINE SQUASHES PERMANENT WITHOUT REMOVAL OF THE COVER SLIP¹

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ABSTRACT.—Two modifications of Bridges' alcohol vapor method for making smear and squash preparations permanent are described. The variation of more general significance permits euparal to be applied without removal of the cover slip. Thus, possibility of loss, distortion or overlapping of desirable material is eliminated from this phase of preparation. The second modification is a reduction in duration of the alcohol vapor treatment. Although the latter alteration in schedule has no particular value in connection with material of many genera, it allows the vapor method to be applied to tissues of *Nicotiana* and may be useful in dealing with material of some other genera.

To supplement a technic for obtaining whole embryo sacs in aceto-carmine squashes of *Nicotiana* ovules (Bradley, 1948), a method was required that would enable preparations to be made permanent without removal of the cover slip. Since ovule squashes contain a preponderance of somatic cells, removal and replacement of the cover slip, as in the procedures commonly used, frequently cause superposition of embryo sacs and somatic cells, in addition to the loss of valuable material and the distortion of cell peripheries liable to occur in any preparation when the cover slip is removed.

Bridges' (1937) alcohol vapor method for making smear and squash preparations permanent had hitherto been unsuccessful when applied to tissues of *Nicotiana* species, because of excessive darkening of the cytoplasm. However, since Bridges' method offered the best possibilities for obviating cover slip removal, variations were sought, and found, to adapt that technic to the requirements.

The modifications reported here are applicable to aceto-carmine squashes of anthers and root tips as well as to those of ovules. Although they have not been tested on material fixed and stained by other technics, it is probable that they can be applied after any pro-

¹Contribution No. 120 from the University of California Botanical Garden.

cedure that has been followed successfully by the original schedule of the Bridges method.²

If permanent preparations are to be made of tissues fixed in a formula having a high chloroform content, such as the one used for ovule squashes (Bradley, 1948), as much of the excess fixative as possible should be absorbed from the material with filter paper before post-fixation steps in preparation are begun.³ Otherwise, chloroform is carried into the stain and may not be replaced completely by alcohol in the subsequent vapor treatment. Since chloroform is not readily miscible with euparal, aggregates of refringent granules will form when euparal is applied. Anthers, or small buds from which anthers are to be removed for squashing, should be drained with particular thoroughness, since no treatment with fluids (during which some chloroform would be removed) intervenes between fixation and squashing.

The technic can be applied either to freshly made preparations or to aged temporary ones. In the case of temporary preparations sealed with a mixture such as gum mastic and paraffin, as much of the seal as possible is carefully scraped away from both slide and cover slip. A drop of xylene is spread over the areas formerly covered by the seal and is then blotted up with lens paper, special care being taken to remove xylene and dissolved seal from the line of union of cover slip edges with the slide.

Before a preparation is made permanent, it may require destaining, particularly if the stain contains an abundance of iron or if the preparation is an old temporary one. (Frequently, however, superior differentiation is found in overstained preparations after they are destained.) In destaining, loss of material is reduced if the slide was heated and pressure applied to the cover glass when the preparation was made. To destain, a drop of dilute aceto-carmine (ca. 1 part stain to 5 or 6 parts 50% acetic acid) is placed on the slide and allowed to run around the cover slip and underneath it. The slide is warmed over an alcohol flame (the stain must not be allowed to boil) to speed penetration of the dilute stain to tissues under the center of the cover

²After this paper was submitted for publication, the method was tested on Feulgen squashes of *Vicia* root tips and *Rhoeo* and *Agapanthus* anthers and proved successful in each case.

³Subsequent investigation has shown that excess chloroform can be removed more thoroughly by soaking the tissue in water for 10 to 20 minutes before it is stained. This is particularly advisable when the material is to be stained with aceto-carmine without preliminary maceration or when the Feulgen procedure is to be used. In the latter case the chief purpose of chloroform removal is to increase the intensity of the stain, since chloroform apparently interferes with hydrolysis or staining with basic fuchsin, or both.

slip. Then the slide is placed within the fold of a paper towel and the region over the cover slip is pressed gently to squeeze out excess liquid. The acetic acid will continue to destain for a time, but if, in view of that, the cytoplasm is still too dark, the above procedure can be repeated. The degree of destaining is rarely equal in all parts of the preparation, due to differences in density of material and in the rapidity with which the destaining fluid comes in contact with tissues in different regions.

Before the preparation is mounted in euparal, it must be dehydrated. This is accomplished, as in the Bridges' method, by vapor exchange. The slide is placed on end in a jar that can be covered tightly, the sides and bottom of which are lined with paper towels saturated with 95% ethyl alcohol. The period of time in alcohol vapor varies with the material. Bridges recommended 24 hours or longer, and this schedule is successful with tissues of many genera. However, material of *Nicotiana* species (and perhaps that of certain other genera) requires a much shorter treatment; 4 to 6 hours' treatment is sufficient, and a 24-hour period in alcohol vapor causes the cytoplasm to darken to the extent that preparations become useless.

After an appropriate period of time in the vapor jar, the slide is removed, and to insure complete exchange of fluids, a few drops of absolute alcohol are applied around the cover slip edge, allowed to run underneath, then pressed out. Several drops of diaphane or euparal, diluted with absolute alcohol to the consistency of thin cream are spread around the edges of the cover slip. The slide is placed, cover slip up, on a piece of absorbent paper in a flat, covered container. The paper is moistened with a small amount of absolute alcohol—enough to soften the euparal and allow it to infiltrate the tissue without diluting it to the extent that it flows over the cover slip. If the container is a $10 \times 10 \times 1.5$ cm. petri dish, 10 to 15 drops of alcohol are sufficient.

After approximately 24 hours the slide may be removed. It should be kept at room temperature for 3 or 4 weeks to allow the euparal to dry slowly, since at higher temperatures the rapidly evaporating alcohol may be replaced by air. In case air bubbles should form under the cover slip, the slide may be returned to the second alcohol vapor chamber until the euparal has softened. Then, by locally directed pressure on the appropriate cover slip regions, the air can be forced out to be replaced by euparal.

SUMMARY OF PROCEDURE

- (1) If the preparation is a sealed temporary one, scrape away the seal and remove remaining traces of it with xylene.

- (2) If necessary, destain either freshly made or previously sealed preparations with 5 or 6 parts 50% acetic acid plus 1 part aceto-carmin.
- (3) Place the slide on end in an alcohol-vapor jar and leave it there for 4 to 6 hours.
- (4) Allow a few drops of absolute alcohol to run underneath the cover slip, then press out the alcohol.
- (5) Apply euparal or diaphane all around the edge of the cover slip.
- (6) Place the slide, cover slip up, in an alcohol-vapor jar in which the atmosphere is *not* alcohol-saturated. (A 10×10×1.5 cm. petri dish requires not more than 10 to 15 drops of absolute alcohol.) After *ca.* 24 hours the slide may be removed.
- (7) Allow the euparal to harden at room temperature for three or four weeks.

REFERENCES

- BRADLEY, M. V. 1948. An aceto-carmin squash technic for mature embryo sacs. *Stain Techn.*, 23, 29-40.
- BRIDGES, C. B. 1937. The vapor method of changing reagents and of dehydration. *Stain Techn.*, 12, 51-2.

LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

OETTLE, A. G. A technique for changing from a bright to a dark ground condenser while a slide bearing a watery mount is still under an oil immersion objective. *J. Roy. Micr. Soc.*, 64, 124-8. 1944.

The apparatus consists of a brilliant source of light with an iris diaphragm, a wide aperture reflecting dark-ground condenser such as the cardioid, and an oil immersion objective with an iris diaphragm. The condenser should produce a hollow cone of light where the most oblique rays have a numerical aperture greater than 1.33 and the least oblique rays have a numerical aperture less than the maximum for the objective. Theoretical requirements for this system are fulfilled by the Zeiss-Siedentopf cardioid condenser N. A. 1.21-1.37 and a Zeiss achromatic oil immersion objective rated as 2 mm. (90 \times), N. A. 1.25, with an iris diaphragm by which the N. A. may be reduced to 0.55.

Good quality slides are essential. Four steps in the technic are given. With this rapid technic changing from bright to dark ground, illumination is accomplished without disturbing the slide under oil immersion.—*Paul G. Roope.*

PHOTOMICROGRAPHY

FROOME, K. D., and JARRET, B. A. The instantaneous photomicrography of living micro-organisms. *J. Roy. Micr. Soc.*, 64, 136-46. 1944.

The camera, with its shutter and body, mechanical timer, electric timer and electric shutter completely described in this article, overcomes the difficulties occurring in the conventional micro-cameras. The following new features are installed in the camera to overcome these drawbacks: (1) Ciné-film is used decreasing the exposure time, thus eliminating blurring by a rapidly moving object. (2) The principal feature of their camera lies in the shutter, which is electrically operated, the timer, both mechanical and electrical, being separate from the camera, thus eliminating vibration by the old methods. (3) The timer is released by foot, thus allowing free use of both hands. (Shutters of normal design were hand operated.) (4) Maximal light reaches the film because the shutter itself is a partial mirror. By inclining the shutter a convenient percentage of light is reflected into an auxiliary viewing eye piece up to the instant of exposure. Exposure time is thus cut down.

Applications of the camera are given. The chief value lies in the comparative ease with which it is possible to take photographs of moving microscopic life.—*Paul G. Roope.*

RAEBURN, L. T. Side focusing eyepiece for small micro-cameras. *J. Roy. Micr. Soc.*, 64, 147-8. 1944.

A concise description is given, illustrated by two figures, of a camera with a built-in side focusing eyepiece. By the method described, the following difficulties are overcome: (1) rigidity of the apparatus with its vibration; (2) the necessity of focusing on ground glass or plane glass by means of a hand magnifier.

The box attachment to the camera is of such a nature that by merely swinging a mirror (housed in the box) to a vertical position, one is able to proceed with the taking of the photograph.—*Paul G. Roope.*

MICROTECHNIC IN GENERAL

BARGHOORN, E. S. Use of phenol formaldehyde and vinyl resins in sealing liquid mounting media on microscope slides. *Science*, 106, 299-300. 1947.

A method is outlined for using phenol formaldehyde and vinyl resins for sealing glycerol and lactic preparations on microscope slides, the former being indicated as superior to sealing compounds now in use. Details for thinning, application of the method and results of aging are described.—*T. M. McMillion*.

HARTLEY, W. G. A variable phase-contrast system for microscopy. *Nature*, 159, 880-1. 1947.

The author describes a variable phase-contrast system based on refracted and dioptric rays which are polarized at right angles to each other. A path difference is introduced by passing these rays through a bi-refracting screen, which produces the effect of different optical thicknesses because its refractive index is different for two perpendicularly polarized rays.—*C. Randall*.

VAN CLEAVE, HARLEY J., and ROSS, JEAN A. Use of trisodium phosphate in microscopical technique. *Science*, 106, 194. 1947.

The author discusses the use of dilute Na_3PO_4 solution, usually 0.25%, for softening and increasing the permeability of dried or resistant material. General discussion of the use of the solution on acanthocephalans preliminary to staining and mounting is given, with indications that the reagent is superior to other detergents.—*T. M. McMillion*.

ANIMAL MICROTECHNIC

ASH, J. E. Technical methods in use at the Army Institute of Pathology. *J. Tech. Methods*, 26, 1-59. 1946.

The entire issue is devoted to a presentation of the details of all technics used in the preparation of tissue at the Army Institute of Pathology. General procedures for fixing, embedding, and staining tissues are described plus many special technics for particular tissues. Staining methods for connective and nerve tissues, microorganisms, blood, and cell products are given. Also included is a section on the preparation of museum specimens.—*C. Randall*.

CALLAN, H. G., and MONTALENTI, G. Chiasma interference in mosquitos. *J. Genetics*, 48, 119-34. 1947.

Satisfactory preparations of testes of mosquito larvae or pupae were obtained as follows: Dissect out under a binocular microscope; fix for a few minutes in a mixture of 8 parts absolute alcohol to 1 part glacial acetic acid; stain for about 5 min. in iron aceto-carmin; squash lightly under a cover slip.—*V. Karanagh*.

CHU, C. H. U. Staining of nerve endings in mouse epidermis by Feulgen's nuclear reaction. *Science*, 106, 70. 1947.

An easier and more reliable method for staining nerve endings in mouse epidermis by Feulgen's nuclear reaction is described. The essential technics employed are those in regular use for other purposes, reference being made to standard handbooks of microscopic technic. Reference is also made to staining nerve fibers by Schiff's reaction.—*T. M. McMillion*.

CLARK, A. E. A method for staining grossly fatty tissues with Scharlach R. *J. Path. & Bact.*, 59, 337-8. 1947.

To avoid bothersome stickiness of frozen sections from grossly fatty kidney and liver, gelatin embedding was found efficacious in allowing the preparation of flat sections. The following procedure was used: After formalin fixation, wash tissue in running water 24 hr. Soak in 12.5% aqueous gelatin at 37°C. for 24 hr.; transfer to 25% gelatin for another 24 hr. Embed in 25% gelatin and refrigerate to set. Place the block in 5% formalin for 24 hr. Trim the gelatin block to within 1 mm. or so of the tissue and cut sections 10-15 μ on the freezing microtome. Transfer sections from knife with a soft brush or with a finger moistened with 50% alcohol to a dish containing 50% alcohol. Transfer the sections to 0.5% gelatin for a few seconds. Pick up sections from the gelatin solution on clean glass slides; a finely drawn glass rod is useful. Drain off excess solution but do not let sections dry.

Place slides in a staining trough over formalin at 37°C. for at least 1 hr. The

slides must not rest in the formalin. Immerse the slides in dilute formalin and leave until ready to stain.

Staining: Rinse sections in distilled water. Transfer to 70% alcohol. Stain in Scharlach R (saturated solution Sudan IV in equal parts of alcohol and acetone) for 3-5 min. Rinse thoroughly in 70% alcohol, then in distilled water. Stain in Ehrlich's hematoxylin for 10 min. Place in running water till blue. Differentiate in acid alcohol. Treat again in running water.

Mount in glycerol jelly or in G. W. Moore's modification of Farrant's mounting medium, composed of: picked gum arabic, 100 g.; cane sugar, 50 g.; distilled water, 300 ml.; thymol, 0.2 g.; glycerol, 25 ml. Dissolve the gum arabic and cane sugar by steaming. Cool, clear with the white of 2 eggs and filter through muslin. Add thymol and finally the glycerol.—*S. H. Hutner*.

CROOKE, A. C. A rapid supra-vital staining method for assessing the viability of human spermatozoa. *Nature*, 159, 749. 1947.

The following method may be used for supra-vital staining of human spermatozoa: Place 4 or 5 drops of seminal fluid on a clean slide, add one drop of stain and mix well; let stand 2-3 min.; withdraw a drop and make a thin smear on a clean slide; let dry in air and then fix for 15-20 sec.; dip slide in alcoholic iodine and into 90% alcohol; allow to dry; counterstain 15-30 sec.; rinse in water and differentiate with care in 90% alcohol.

The reagents are prepared as follows:

Supra-vital stain: 50 ml. distilled water; 1 g. Revector soluble blue 706; 1.5 g. glucose; 0.1 g. NaCl; 0.3 g. Na_2HP_4 , hydrated; 0.005 g. KH_2PO_4 . This solution must be made up fresh or stored in 1 ml. ampules and autoclaved.

Counterstain: 1% neutral red in distilled water.

Fixative: 1 vol. saturated solution of HgCl_2 in distilled water; 1 vol. absolute alcohol; followed by 90% alcohol containing a few drops of iodine in KI to give a straw-colored solution.

The nuclei of the spermatozoa which were dead when the seminal fluid was mixed with the stain are blue or purple; those that were alive are clear red, and a few non-nucleated forms remain unstained.—*C. Randall*.

GOLDSCHMIDT, ELIZABETH. Studies on the synapsis in salivary chromosomes of hybrid *Chironomus* larvae. *J. Genetics*, 48, 194-205. 1947.

Salivary glands of prepupae were dissected out in a drop of Ringer's solution, left for 10 min. in 45% acetic acid, and stained 10 min. in aceto-carmin before crushing. Permanent preparations were obtained by transferring the smears through 95% alcohol into Venetian turpentine.—*V. Karanagh*.

R. D. LILLIE. Reticulum staining with Schiff reagent after oxidation by acidified sodium periodate. *J. Lab. & Clin. Med.*, 32, 910-12. 1947.

The following procedure is recommended: Deparaffinize thin sections (5 μ or less) and bring up to 100% alcohol as usual. Soak 5 min. in 1% collodion in 50:50 ether and alcohol, drain and harden 5 min. or longer in 80% alcohol. Wash 5 min. in running water and immerse in sodium periodate (Na_2IO_3 1.0 g., 70% HNO_3 0.5 ml., and distilled water 100 ml.) for 10 min. Wash 5 min. in running water. Immerse 15 min. in Schiff reagent (place 1 g. basic fuchsin in 100 ml. of distilled water at 95°C., filter, cool and add 2 g. HN_2SO_3 and 20 ml. of $N/1$ HCl). Transfer to 3 successive baths of 0.5% NaHSO_3 . Wash 10 min. in running water. Stain 5 min. in Weigert's iron hematoxylin. Counterstain 1 min. in saturated picric acid or 1% orange G. Dehydrate with alcohols and clear with xylene. All reticulum, subepithelial basement membrane and the stroma of cerebral capillaries stain bright purplish red. Collagen stains pink. Cartilage matrix stains red-purple, epithelial mucin reddish purple to almost violet, and glycogen dark red purple to almost black.—*John T. Myers*.

SHEEHAN, H. L., and STOREY, G. W. An improved method of staining leucocyte granules with Sudan black B. *J. Path. & Bact.*, 59, 336-7. 1947.

This method appears somewhat more reliable than those previously described, e.g. Discombe (*abs. Stain Techn.*, 22, 121, 1947; compare with Bailiff and Kimbrough, *abs. Stain Techn.*, 22, 120, 1947).

The dry new or old blood film is fixed with formalin vapor by placing it for 5-10 min. in a closed vessel with 4% formalin. It is stained in a solution prepared as follows: Dissolve 0.3 g. Sudan black B in 100 ml. absolute alcohol. Prepare a buffer solution by dissolving 16 g. of phenol crystals in 30 ml. of absolute alcohol; add this phenol solution to 100 ml. of water in which 0.3 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ is dissolved. Mix 40 ml. of the buffer solution with 60 ml. of the dye solution. The dye must be completely dissolved before mixing. Filter the final solution by suction. The stain solution can be used for several weeks.

The fixed film is immersed in the buffered stain solution in a covered jar for 10-60 min. Slides are washed in absolute alcohol or 70% alcohol for a few min., and are then washed in water. They are counterstained with Giemsa (Gurr's Improved Giemsa R. 66) diluted 1:10 in neutral solution. The blue tint of the plasma and erythrocytes is removed by differentiation 30-60 sec. in 0.2% aqueous KH_2PO_4 solution. The granules stain intense black.—S. H. Hutner.

VERDCOURT, B. An introduction to the study of radulae. *Microscope*, 6, 35-9. 1946.

An undetailed review of staining methods for radulae of molluscs is given by the author. Enamel may be removed by a decalcifying agent and the unhardened teeth stained with an acid dye. More popular methods keep the enamel and modify the chitin, usually by oxidation with KMnO_4 , decolorization with dilute oxalic acid. The radula is then washed with water and stained in dahlia. Chrysoidin, hematoxylin, and chlorazol black E, the latter in either 70% methyl alcohol or saturated aqueous solution, may also be used.

Canada balsam and Euparal are the best mountants. Glycerin jelly is not recommended because of its low refractive index and decalcifying properties.—C. Randall.

PLANT MICROTECHNIC

BHADURI, P. N., and BOSE, P. C. Cyto-genetical investigations in some common cucurbits, with special reference to fragmentation of chromosomes as a physical basis of speciation. *J. Genetics*, 43, 237-56. 1947.

Root tips were fixed in Levitsky's fixative, equal parts of 1% chromic acid and 10% formalin or 2 parts 1% chromic acid to 3 parts 10% formalin. Flower buds were pretreated with Carnoy's fluid as modified by Semmens (1 vol. chloroform, 3 vol. alcohol, 1 vol. acetic acid) before fixation in Belling's modification of Navashin's fixative. Paraffin sections stained well with Feulgen light-green or Newton's crystal violet iodine but not with aceto-carmin. Mordanting slides with 1% chromic acid after KI-I treatment improved staining. Several other slight modifications are suggested for specific cucurbits.—V. Karanagh.

VON ROSEN, GOSTA. A rapid nigrosine method for chromosome counts applicable to growing plant tissues. *Nature*, 160, 121-2. 1947.

The author recommends the following method for chromosome counting in roots, leaves, and flower-buds: Fix 24 hr. in 1 part concentrated acetic acid plus 2 parts 95% alcohol. (Roots should be fixed in a cooled fluid). Replace fixing fluid with a cooled mixture of 1 part concentrated HCl plus 2 parts 95% alcohol. (Roots should be treated 8-10 min. For leaves the character of tissue determines concentration and time: *Beta*, 1:2 for 7-8 min.; *Pisum*, 1:4 for 5 min.; *Pyrus* and *Betula*, 1:1 for 10 min.; flower-buds, 1:10 for 5-8 min.). Replace loosening fluid with distilled water and keep in a cool place 15-30 min. Place specimen on object glass and add one drop of staining solution given below. Stain roots 1-2 min.; leaves of *Pisum* 30 sec.; those of *Beta* 40 sec.; *Pyrus* and *Betula* 45-60 sec. Remove excess stain with filter paper.

The staining solution is prepared as follows: dilute 50 ml. concentrated acetic acid with distilled water to 100 ml.; heat to boiling-point; add 4 g. spirit-soluble nigrosin with continuous stirring; boil 3-5 min. to a weak tarry consistency; cool and filter immediately. Keep the solution in a closed glass vessel at room temperature for about 10-14 days before use. For buds only a 3% solution is used.

When this technic is used, chromosomes are stained quite black and the plasma a faint grey.—C. Randall.

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MORDANTING PLANT TISSUES¹RICHARD A. POPHAM, *Ohio State University, Columbus, Ohio*

ABSTRACT.—Selected current ideas on the mordanting of plant tissues are presented in the hope that they will be of practical assistance, especially to the beginner. The nature of the mordanting process and the results which may be expected following mordanting are briefly described. Specific technics are presented for the mordanting of natural dyes, synthetic dyes generally, the basic synthetic dyes and the acid synthetic dyes.

INTRODUCTION

Since an understanding of mordants and mordanting is of primary importance to the microtechnician, one would expect to find a great deal of literature dealing with the subject. As a matter of fact, there has been very little written which applies to general histological staining of plant tissues. Information which is available is not only incomplete but widely scattered and is mostly presented in the discussions of specific stain schedules. It was thought, therefore, that a summary of some of these data might be of practical assistance to the beginner and might stimulate additional research in the field by those qualified to do it.

Technicians have been forced to study the mordanting of tissues for natural dyes because without a mordant, they stain very poorly or not at all. However, there are only three groups of natural dyes of importance to botanists, cochineal and its derivatives, brazilin, and hematoxylin. Only hematoxylin and carmine are used extensively. The immediate urgency and obvious incentive for conducting similar studies in connection with synthetic dyes are absent. Among the numerous synthetic dyes, the technician usually has a choice of several which may be employed to stain a particular part without the necessity of mordanting. It is probably these circumstances which account for the paucity of literature on the subject of mordanting plant tissues. Even though many synthetic dyes do not as a rule

¹Papers from the Department of Botany, The Ohio State University, No. 501.

require a mordant, experienced technicians know that better results can often be obtained by using one. Mordanting frequently reduces protracted staining periods to a few minutes, produces more brilliant results, and permits the technician to stain positively or negatively charged cell parts with acid or basic dyes almost at will. In addition, unintentional mordanting by killing solutions sometimes makes reverse mordanting for synthetic dyes a necessity.

Mordants are usually thought of as chemicals which in some manner prepare cell walls, nuclei, nucleoli, chromosomes, or other cell parts so that they may be dyed by the stain. They may be used in any one of three positions in the stain schedule depending upon the mordant and dye used: (1) preceding the stain; (2) mixed with the stain forming a self-mordanting solution; (3) following the stain. Often more than one mordant is used. In any event it is probable that the mordant penetrates the material to be stained and becomes attached to it (probably an adsorption phenomenon). Initially, simultaneously, or subsequently the dye ion penetrates the object and probably combines chemically with the mordant forming a color compound. It seems possible that some of the so-called mordants simply change the pH of the self-mordanting stain solutions or alter the physical state of the cell part sufficiently to permit adsorption of the dye. Although the physics and chemistry of mordanting remain highly debatable subjects, the effects are positive and can be easily demonstrated by the novice. It is apparent then that a working knowledge of mordanting is an extremely useful tool in the hands of the technician.

NATURAL DYES

Let us consider first the mordanting of natural dyes. Brazilin, a dye of relatively little importance to botanists except as a stain for smears, is usually preceded by a mordant of 1% ferric ammonium sulfate in 70% ethyl alcohol (Capinpin, 1930). The stain most frequently used for smears, carmine (acid), is usually prepared in a self-mordanting solution with salts of different metals. Ferric acetate, ferric hydrate, ferric chloride, ferric hydroxide and sodium tetraborate (borax) are frequently employed (Chamberlain, 1932; Johansen, 1940). When carmine and carminic acid are used for permanent preparations, mixtures containing HgCl_2 may be employed in the dual role of killing solution and mordant.

While chromic acid and mercuric chloride in killing fluids usually mordant tissues for the basic natural dye, hematoxylin, staining is always preceded by a specific mordanting solution or a self-mordanting dye solution is employed. The more commonly used self-

mordanting hematoxylin stains are Delafield's and Harris', in which the mordanting compound is ammonium aluminum sulfate, and Erlich's and Mayer's, in which the mordant is potassium aluminum sulfate. Tissues difficult to stain with Harris' hematoxylin may be transferred from distilled water to a mordant of 1/N HCl (82.5 ml. C.P. hydrochloric acid in 1,000 ml. of distilled water) for one hour. After rinsing in distilled water and staining, it will be found that nuclei and dividing chromatin have stained intensely and sharply. (Harris' Hematoxylin, El Palo Alto News, 2, 6. 1938.)

Of all the mordants, iron alum (ferric ammonium sulfate) is the most widely recognized because of its extensive use in connection with Heidenhain's iron-hematoxylin. The mordant is usually a 2-5% aqueous solution and precedes the stain. If ferric ammonium sulfate (12-24 hours) or ferric sulfate is used, the resulting stain is a deep dark purple. When the tissue is mordanted in ferric ammonium sulfate for a short time (30 min.) or in ferric chloride, the stain is blue. If a black stain is desired, ferrous sulfate or ferric acetate may be used. A copper chloride mordant gives a deep blood red stain while aluminum ammonium sulfate or potassium ammonium sulfate gives high contrast blue results. Chromium aluminum sulfate results in a gray-blue color. In addition to these mordants, tin and uranium salts have been used. Lang (1936) reported better results in obtaining high contrast in the staining of chondriosomes, plastid primordia, and chromonemata when 5 ml. glacial acetic acid and 0.6 ml. sulfuric acid (C.P., Sp. Gr. 1.84) are added to 500 ml. 4% aqueous ferric ammonium sulfate (C.P.). Although Lang cautioned against the use of his mordant for general histological staining, it has been widely adopted by microtechnicians for this purpose, with excellent results. The use of 2% aqueous $ZnCl_2$ as a preliminary mordant for staining newly formed cell walls in the shoot apex with iron-hematoxylin was described recently (Sharman, 1943). Cole (1943) summarizes much of the literature on the mordanting of hematoxylin and gives in addition the results of his own research on the subject.

SYNTHETIC DYES

There are very few chemicals which mordant for both basic and acid synthetic dyes. Killing fluids such as "CRAF" modification of Navashin's, FAA, FPA, and others containing chromic acid, formalin, and osmic acid apparently mordant for most synthetic dyes. A 2-4% formalin solution followed by washing in water may be used before staining, regardless of the killing solution used. A 1% aqueous solution of potassium permanganate (about pH 7) is a powerful mordant for the entire group of synthetic dyes. It should be used

for only a short time (less than 10 minutes) preceding a short staining period in the dye. In the past, anilin water (usually a 2 or 3% aqueous solution of anilin oil vigorously shaken) has been generally used as the solvent for making self-mordanting aqueous synthetic dye solutions. The modern technic is to dissolve the dye in 10 ml. absolute alcohol which is then mixed with 100 ml. 4% formalin giving a self-mordanting stain solution. A filtered iodine solution made by saturating water first with potassium iodide and then with iodine may be used in low concentrations as a mordant following staining. The degree of dilution depends upon the stain used. The mordant should be followed by washing in water.

BASIC SYNTHETIC DYES

Mordants used in conjunction with the synthetic dyes may best be discussed as they apply to those dyes whose color is due to an electro-positive ion of an organic base (basic dyes) or to the remaining synthetic dyes whose color is due to a negative ion of an organic acid (acid dyes). The basic synthetic dyes are sometimes inaccurately referred to as "non-cytoplasmic stains" because many are used to color nuclei, chromosomes, and lignified walls. Acid mordants such as 4% aqueous silicotungstic acid, chromic acid killing fluids, and tannic acid used previous to staining, act as general mordants for this group of dyes. Tannic acid, in addition to mordanting nuclei particularly well for safranin, has also been used with success on cellulose cell walls (cotton) previous to staining with basic dyes. It is probably due to these two facts that tannic acid has been found useful in mordanting shoot tip sections (Foster, 1934; Northern, 1936; Sharman, 1943). Flemming's killing fluid, containing both osmic and chromic acids, may be used as the original killing fluid or may be used for 24-72 hours following preservation of tissues in Bouin's or Carnoy's. In either case, Flemming's mordants chromatin for staining with basic dyes.

Killing fluids such as "CRAF" modification of Navashin's and FAA or FPA prepare nuclei and chromosomes especially well for staining with safranin. If aqueous or alcoholic solutions of safranin are desired, self-mordanting stains may be prepared in three ways: (1) by adding 0.5 ml. glacial acetic acid and 5 ml. 4% aqueous aluminum ammonium sulfate to each 100 ml. of the dye mixture; (2) by mixing 50 ml. 5% aqueous anilin water with 50 ml. 1% safranin; (3) according to Johansen's (1940) superior formula in which 2% formalin is incorporated as the mordant. A solution of 0.5% picric acid in 95% ethyl alcohol applied after staining in safranin, and followed by washing in water, acts both as a destaining agent and as a mordant.

Weak alcoholic picric acid or iodine solution (1 g. iodine and 1 g. potassium iodide in 100 ml. 80% ethyl alcohol) mordant for basic violet dyes when applied *after* staining. Newcomer (1938) suggests a mordant of 1% aqueous chromic acid (30 min.) followed by washing in tap and distilled water. After staining in 1% aqueous crystal violet (1 hour) the material is treated for 30 sec. in a second mordant (1 g. iodine plus 1 g. potassium iodide in 100 ml. 80% ethyl alcohol).

ACID SYNTHETIC DYES

Acid synthetic dyes usually serve as cytoplasmic and cellulose wall stains. Mordants such as 2-4% barium chloride, aluminum, or chromium salts followed by slightly alkaline washes of ammonium hydroxide or sodium carbonate are most frequently used. A self-mordanting anilin blue may be prepared by adding small quantities of aluminum ammonium sulfate to the dye solution. Eosin and erythrosin self-mordanting stains may be prepared by adding a trace of acetic acid to the solvent. Tissues difficult to stain with alcoholic fast green FCF, especially following iron-hematoxylin, often dye perfectly when first mordanted with a mixture of one part saturated citric acid in 95% ethyl alcohol and one part 95% ethyl alcohol. Excellent results are obtained when nucleoli are stained with a saturated solution of light green SFY in 90% ethyl alcohol, to which 2 drops of anilin oil are added per 100 ml. stain solution. In addition, Semmens et al (1939 and 1941) suggest mordanting 1 hour or longer in a saturated aqueous solution of sodium carbonate (filtered). The mordant is followed by an 80% ethyl alcohol wash and 10 min. in the stain solution.

The foregoing results emphasize the complexity of the mechanism of staining and show how hard it is to explain it by either a chemical or a physical theory alone. The fact that each of the described mordants will not effect every tissue as expected is pretty good evidence in itself that the staining mechanism is more complex than either group of theorists would have us believe. The effect of the mordant as well as the solvent on the isoelectric point of the cell parts is a factor which deserves much more critical study than it has received to date.

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PHASE MICROSCOPY IN BACTERIOLOGY¹

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ABSTRACT.—The function and use of the phase microscope is described for controlling the contrast in the image and making visible unstained, living microorganisms and cytological details within them. The microscope may be used to examine unstained, growing cultures in Petri dishes, even with the oil immersion lens. Flagella are shown on the living spore of *Ashbya*. Since microorganisms show sharp edges under the phase microscope, measurement of unstained living cells is now possible. *B. megatherium* was found to average 1.0μ in width and *B. cereus* 1.1μ in width with very small variation. Observations on locomotion are like those reported by Pijper. Stained preparations of low contrast may be seen with considerably enhanced contrast by phase microscopy.

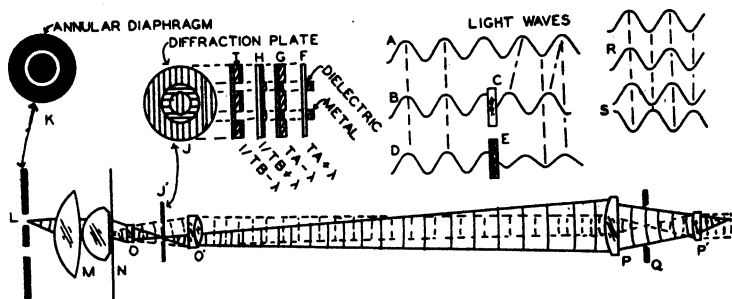


FIG. 1.—Light path through the Spencer Phase Microscope.

Microorganisms, living and unstained, can scarcely be seen with the usual brightfield microscope. Increased contrast may sometimes be attained from closing the diaphragm of the microscope condenser to obtain a narrow cone of illumination. Other times the decrease in resolution from this procedure prevents seeing the detail before enough contrast is developed. The phase microscope shows the organisms in sharp outline and with internal detail at full resolution. Phase microscopy utilizes optical path differences within the instrument to increase or decrease, or to reverse and increase or decrease

¹Based on reports to the 1946 and 1947 annual meetings of the Society of American Bacteriologists

contrast in the image. It is especially useful with unstained living cells, but when the contrast of stained material is not great, it is often possible to enhance visibility, especially of small details.

Optical path differences (optical path = thickness \times refractive index) alter the speed of light passing through them. A greater path as C, Fig. 1, slows the wave B with respect to A which did not pass through the higher index region, but the eye and the photographic emulsion are insensitive to phase differences. Absorbing media, like E, decrease the amplitude of the waves without changing their phase relations and such differences are visible and photographable. The change of phase into amplitude differences is accomplished by the phase microscope.

An annular diaphragm L is placed at the front focal plane of the condenser. Light from any point L, likewise from every point in the annulus, passes through the condenser and comes to a focus somewhere in the objective system. In the 16 mm. Spencer objective this occurs between the lens system at J' and a bright ring of light is formed there as a small image of the opening in the diaphragm K. At this point is placed a diffraction plate J, which is a piece of optical-grade glass with a thin layer of metal, or of a dielectric, or both, large enough to cover the conjugate or image area. Or, the phase-altering materials may be placed over the rest of the disc on the complementary area.

When a specimen is placed in focus at N, a part of the light is deviated by it and most of this deviated light from the specimen does not pass through the conjugate area. The diffraction plate can affect differentially the deviated and undeviated light from the specimen and the undeviated light from the background depending on its composition. Four types of diffraction plates are possible, Fig. 1, F, G, H, and I, although H has found little use in biology. An A+ plate, like F, retards and partially absorbs the undeviated light without affecting the light deviated by the specimen and gives bright contrast, *i.e.* regions of greater optical path will appear brighter than regions of the same size of lesser path. The A- diffraction plate, G, partially absorbs the undeviated light and retards the deviated light, giving dark contrast. The B-type, I, does not affect the undeviated light but partially absorbs and retards the light deviated by the specimen and also gives dark contrast. The latter type is useful with supervitally and other stained material.

The absorbing metal layer may be made of any desired transmission and the retarding layer depends on its optical thickness for its action. Of the many possible combinations a few are of greatest use in reveal-

ing microorganisms. The most generally useful plates are a $0.2A + 0.25\lambda$ for bright and $0.2A - 0.25\lambda$ for dark contrast. These absorb about 80% of the undeviated with respect to the deviated light and retard (+) or advance (-) the phase relations of the undeviated light with respect to those of the deviated light. When all the light comes together to form the image, rays like R, Fig. 1, add together to give a brighter image while those of the same amplitude but of opposite phase destroy each other, giving no light and appear black. Other combinations provide grays. Thus, the invisible phase changes from transparent specimens are changed to visible amplitude differences. Further explanation requires mathematical analysis and is available elsewhere with the history of the method (Bennett, 1946; Bennett *et al.*, 1946). Richards (1947) summarized applications of phase microscopy in biology.

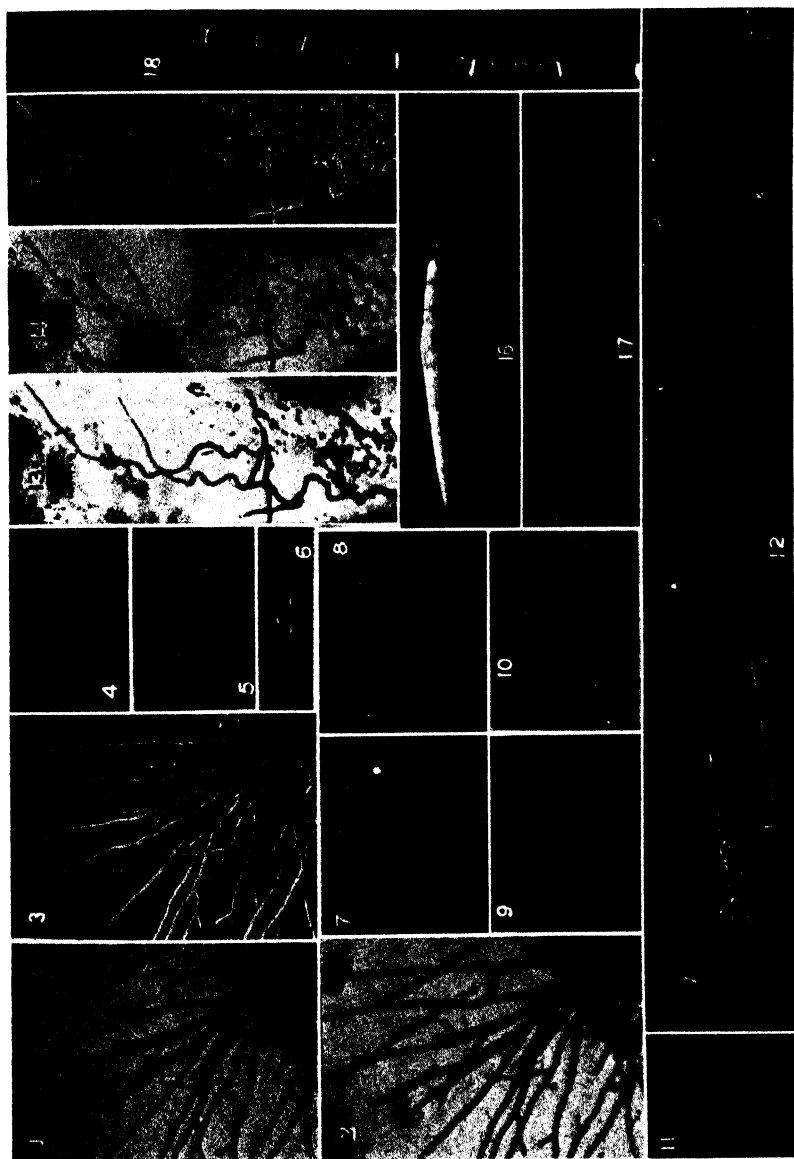
For the smaller organisms like bacteria and molds decreasing the transmission gives greater contrast and the $0.14A + 0.25\lambda$ and $0.14A - 0.25\lambda$ diffraction plates have proven useful. For the smallest detail within the cells a still higher contrast of $0.07A + 0.25\lambda$ is helpful, especially in bright contrast. Some of the larger bacteriophages have been seen with a $0.1A + 0.25\lambda$ diffraction plate (Hofer & Richards, 1945). Yeast show better in dark contrast with a $2.5B - 0.33\lambda$ plate. Other plates are required by special cases. The diffraction plates may be obtained for the 16, 8, 4, and 1.8 mm. objectives. The Spencer equipment consists of a condenser with separate centering screws for the condenser and for the annular diaphragm, an objective with appropriate diffraction plate and a telescope for centering. In use the microscope is focused on the specimen, the telescope placed in the eyepiece tube in place of the ocular and the condenser is centered to the diffraction plate. The annular diaphragm is inserted into the condenser and centered to the plate, the telescope is removed, the ocular replaced, and the instrument is ready for use. A good research type microscope lamp with a focusable condenser is desirable for best results. When the greatest contrast is desired monochromatic light is desirable. It is not difficult to use the equipment and full directions are supplied.

No special preparation methods are required, although thinner are better than thicker preparations. Individual bacteria, Fig. 5-11, show clearly in bright or dark contrast. The margins of the cells are not diffuse regions of diffraction as with the brightfield microscope, Fig. 2, but sharp enough for measurement. Measurements of actively growing 20-hour broth cultures from pictures like those of Fig. 5-11 give the following data: *Bacillus megatherium*, width 1.00μ , $\sigma = 0.06\mu$,

PLATE 1.

Since detail is lost through reduction and reproduction of the figures, copies of some of these will be made available through the Visual Aids Committee of the Society of American Bacteriologists.

1. *Cladosporium* culture growing on agar in a Petri dish. Brightfield microscope. 70 \times .
2. Same as 1 with phase microscope with dark contrast. 70 \times .
3. Same as 1 with phase microscope with bright contrast. 70 \times .
4. *B. megatherium*, living, unstained from a 20-hour broth culture. Brightfield microscope. 900 \times .
5. Same as 4 with phase microscope in bright contrast. 900 \times .
6. Same as 5 another field.
- 7-8. *B. cereus*, living, unstained, from a 20-hour broth culture. Phase microscope with bright contrast. 900 \times .
- 9-10. Same as 7-8 with phase microscope with dark contrast. 900 \times .
11. *B. cereus* with phase microscope with increased bright contrast. 900 \times .
12. *Cladosporium* growing in agar in Petri dish. Phase microscope with bright contrast. 800 \times .
13. *Monilia* growing in agar in Petri dish. Phase microscope with dark contrast. 75 \times .
14. Same as 13. Brightfield microscope. 75 \times .
15. Same as 13. Phase microscope with bright contrast. 75 \times .
16. *Ashbya gossypii*, living, unstained. Phase microscope with bright contrast. 1,600 \times .
17. Same as 16. Brightfield microscope. 1,600 \times .
18. *Cladosporium*, same as 12. Phase microscope in bright contrast. 800 \times .



number measured, 25; *Bacillus cereus*, width bright contrast phase micrographs, 1.10μ , $\sigma=0.07\mu$, number, 40, dark contrast phase micrographs, 1.05μ , $\sigma=0.05\mu$, number, 35. Measurement was somewhat easier with the dark contrast as the edge of the image was sharper on the photomicrographs and the standard deviation (σ) was a little less. This increased sharpness may have resulted from the Eberhard effect occurring during the development of the film. The difference between the measurements in bright and dark contrast is no more than 1σ and is not significant. This is important to the theory of phase microscopy and confirms the theoretical derivations (Bennett *et al.*, 1946). Both organisms were obtained through the courtesy of Dr. A. W. Hofer from the New York State Agricultural Experiment Station. Brownian and other motion in the preparations was stopped by taking the photomicrographs with exposures of about $1/35,000$ second with a special flash tube (Richards, 1947c).

Comparison with other measurements is difficult owing to the variation within strains and during the life cycle. Knaysi (1945) has reported the width of *B. cereus* to average 1.68μ on living cells, 1.60μ by his cell wall method and 0.61μ when using Meyer's methylene blue method. Knaysi (1944) reported the average width of 12 strains of *B. megatherium* to be from 0.9μ to 1.7μ when measured on heat-fixed, gentian violet stained bacteria. Dubin & Sharp (1944) found measurement difficult on the living cells with a brightfield microscope. The average width to the outer edges of the diffraction zones was reported 1.665μ , to the middle of zones was 1.185μ and between the inner edges of the zones was 0.725μ . Their electron micrographs measured 1.398μ for the total bacterial substance and 1.207μ ($\sigma=0.15$) for the inner bacterial substance.

The living bacteria show sharp edges with the phase microscope and little evidence of other than a very thin cell wall. The protoplasm seems to fill the cell uniformly and to show structural differentiation. Further study should reveal whether the inner and outer regions, shown by the electron microscope, are normal to the organisms or due to desiccation of the preparations. Further measurements of other strains during the life cycle of the same strain should add to knowledge and aid in evaluating the nature and extent of size variability. The measurements here reported from phase micrographs are uncertain by about 0.05μ . The pictures were made with a 1.8 oil immersion objective with a $0.2A+0.25\lambda$ diffraction plate for bright and a $0.14A-0.25\lambda$ plate for dark contrast. It might be noted that the uncertainty of measurement reported is less than the usual figure of 0.2μ for white light. The latter value is for the

separation of very small points and does not apply to the edge of an extended line. Since the edges of bacteria do not show clearly in unstained preparations with the brightfield microscope and staining methods (even negative staining) usually alter the size of the organisms, the phase microscope offers the most effective method for measuring the size of normal bacteria living in a natural medium (Richards, 1946).

The size and shape of the cells show equally with bright as with dark contrast. Intercellular details are revealed more clearly by bright contrast probably because the eye is more sensitive to small bright objects on a dark background. The presence of details too small to be resolved as to size and shape may be revealed in bright contrast when the illuminant is sufficiently intense. Septa in mold filaments show well, Fig. 12 and 16. With high contrast small bodies are seen within the cells, Fig. 11. Some of the unstained *B. cereus* showed paired bodies in the photomicrographs of nonsporulating cells, top center of Fig. 8, somewhat similar to those figured by Robinson (1944).

A large flagellum may be seen on the living spore of *Ashbya gossypii*, Fig. 16, that is too thin to show with brightfield, Fig. 17. This is an illustration of the resolution of the brightfield microscope decreasing faster than contrast builds up as the microscope condenser is closed. Since the Spencer phase objectives are used at full aperture this does not happen. Both theory and practice have shown that the addition of the phase equipment does not reduce resolving power appreciably and, in addition, there is less likelihood of spurious images with phase microscopy. The resolving power of the phase microscope is not increased, but visibility of detail is enhanced.

Living cultures (unstained) may be studied to advantage in Petri dish cultures when the bottom of the dish is reasonably flat. The usual top lens of the condenser is removed and one with a thinner lens is used in its place. Cultures not thicker than about 4.5 mm. may then be examined. The growth may be studied and measured with low power, Fig. 1-3 and 13-15. The greater sharpness of both the bright and dark phase over that of ordinary bright field is apparent. If a cover glass is placed over the specimen the 4 mm. objective may be used without the lens fogging, and with oil contacts to the condenser and to the objective the full detail shown by the oil immersion objective may be seen in Petri dish cultures, Fig. 12 and 18. Fig. 12 shows the granular protoplasm at the growing tip and the septation and vacularization in the mycelium nearer the center of the colony. Rough colonies of *Brucella abortus* furnished through

the courtesy of Prof. Werner Braun (Univ. of Calif.) showed granular, pseudopod-like projections at the growing edge while the corresponding smooth colony edge was uniform and continuous.

Individual bacteria may be seen at the growing edge with the phase microscope and motion picture studies should add appreciably to our knowledge of behavior and the mechanism of growth. Both the myceloid and yeast-like cells of the *Monilia* show as they grow into the agar medium, Fig. 13-15. Living cells of *Streptomyces griseus* show spores forming at both ends. Life history studies may be facilitated.

With thicker cultures, or those in Carrel flasks, but which are sufficiently transparent, the front lens of the condenser is removed and a different annular diaphragm is used, making possible the study of preparations up to about 18 mm. thick with the 16 mm. objective.

Little study of capsulated forms has been made as most of these are too pathogenic for the available facilities. A preparation of *Aerobacter aerogenes* in glycerin jelly showed a somewhat indefinite halo around the cell. Another capsulated form suggested a vague region around it. It may be that in the living cell the capsule is not so definite, or it may well be that they were not observed in the proper medium. Best vision with the phase microscope occurs when the optical path differences are not great and the organism is in a medium of nearly the same refractive index. The contrast may be varied by changing the index of the medium within limits tolerated by the organism which extends the possibilities of the method.

The smaller swimming organs like the classical pictures with stained material have so far not been seen. *Bacillus subtilis* moves too fast for direct vision and some were placed in a medium with some *Methocel* dissolved in it. This slowed the motion and at the posterior of the organism was seen a small swimming mass which resembled the mucous twirls figured by Pijper (1946, 1947). Rod-shaped bacteria were seen to swim with a sinuous or undulatory movement and the author's observations with the phase microscope agree with those made by Pijper with the dark field microscope. The phase microscope has an advantage in showing a sharper outline and more detail within the cell than does the dark field microscope and can be used with less intense illumination making cinephotomicrography less difficult than with dark field. Fruitful studies along these lines are now possible.

Bacteria may be seen with the phase microscope in many products without staining. However, small coccus forms of the same size as fat globules may not be distinguished from each other in milk. Other

forms are readily seen and counted. When the refractive indices and sizes of the bacteria and other material are not the same they can be differentiated even when of the same size and shape. For spirochaetes in pus the dark field microscope is preferable as the phase microscope increases the visibility of the pus as well as the bacteria, so that they are not as readily found. This may be true also for tubercle bacilli in sputum. Some bacteria show well in unstained fresh or fixed tissue.

When the contrast of stained bacteria is not good it is possible to increase it by the use of the proper diffraction plate. For super-vitally and Feulgen stained preparations a $1B-0.25\lambda$ plate is best. For other stains the A- plate gives greater contrast and with some red stains (aceto-carmin and carbol-fuchsin) the bright phase $0.07A + 0.25\lambda$ plus a red Wratten "A" filter provides excellent contrast between the organisms and back ground. Filters of similar shade have proven better with phase microscopy than the contrasting complementary colored filters commonly used.

Phase microscopy is only a few years old and much experimenting will be necessary before all its potentialities are known. It is especially well suited to the study of unstained living organisms and should yield much knowledge when applied to the problems of bacteriology. A few applications have been suggested. European results are likewise encouraging (Bosshard, 1944; Richards, 1947a). Like all new methods some experience is necessary for efficient use and interpretation of the results.

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HISTOLOGICAL TECHNIC FOR CEREBELLAR CLIMBING AND MOSSY FIBERS

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ABSTRACT.—In order to avoid disadvantages attendant upon the use of fresh frozen sections, or of block impregnation with silver, in staining climbing or mossy fibers of the cerebellum, Rio Hortega's double impregnation method for nerve fibers is useful. This consists of prolonged formalin fixation prior to cutting frozen sections (which thereafter are easier to cut) and preliminary treatment with ammoniacal aqueous and alcoholic washes, mordanting in pyridine silver, and treatment with pyridine-silver-carbonate. Following this, sections are handled individually through one of several reduction methods after which they may be directly mounted or gold toned.

The usual staining technic employed for demonstrating the climbing or mossy fibers of the cerebellar cortex is Cajal's block impregnation after fixation in chloral hydrate. Since it is difficult to cut the thin slices necessary for this method from the fresh cerebellum the planes of the finished sections are likely to be uncontrollable. Further disadvantages of block impregnation are the usual unevenness of the stain and, especially serious in experimental situations, the impossibility of knowing from just what part of the cerebellar cortex to take critical material and the impossibility of controlling or repeating the impregnation. With the exception of the last difficulty these disadvantages of block impregnation can be overcome by using Cajal's method (1921 and 1926) for frozen sections (formalin fixation, silver nitrate with pyridine for 2 to 48 hours, alcohol, reduction with formol-hydroquinone) which he recommended for both climbing and mossy fibers. Since the duration of immersion in silver nitrate solution makes control of impregnation difficult, we have preferred Rio Hortega's (1921 and 1942) double impregnation method for neurofibrils and nervous fibers.

TECHNIC

Brains are immersed in 10% neutral formalin (the word formalin is used in this article for an aqueous solution containing from 36-38%

¹Fellow of the Commonwealth Fund of America.

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HCHO) and hardened for a period of at least 15 days. Gross sections of the cerebellum are now cut on a plane, perpendicular to the folla (and thus parallel to the plane of branching of the Purkinje cells). From these blocks frozen sections are now cut at $15\text{--}20\mu$ and these are collected in a dish filled with distilled water alkalized with a few drops of ammonia. They are then treated as follows:

- (1) Rinse in distilled water to get rid of excess of ammonia and free bits of tissue.
- (2) Remove to cold 95% alcohol with 3 drops of ammonia per ml. of alcohol. Warm to $45\text{--}50^{\circ}\text{C}$. for 10–15 minutes. (Alcohol dissolves myelin and ammonia prevents shrinkage. Both favor fiber staining.)
- (3) Transfer to distilled water with ammonia and then wash in three changes of distilled water.
- (4) Mordant in 2% silver nitrate with pyridine (10 to 20 drops for 30 ml. of silver nitrate solution) at $45\text{--}50^{\circ}\text{C}$. When the sections start to take a yellowish color, one or two are removed and the technic is completed on these sections to control the results. (It usually requires about 30 minutes for the sections to assume the light yellow-brown color which they should have before subjection to silver carbonate, step 5.)
- (5) Transfer sections directly to weak solution of Rio Hortega's silver carbonate³ with 5 to 20 drops of pyridine⁴ per 30 ml. of silver carbonate, at $45\text{--}50^{\circ}\text{C}$. The staining dish should be covered with a watch-glass and agitated gently at intervals of about 2 minutes.
- (6) When the sections start to show a light caramel-brown color transfer one section to neutral formalin for control. The following variants are to be tried in the order given, selecting whichever gives the best results when the section is examined:
 - (a) Formalin, 1%.
 - (b) Immerse section in distilled water and turn it several times, very gently, then transfer to 10% formalin. The color of the section will be lighter the longer it is left in the preliminary aqueous wash.
 - (c) Wash thoroughly with distilled water and tone without

³Pure silver nitrate 10% 50 vol.

Pure sodium carbonate 5% 150 vol.

Ammonia water, enough to dissolve precipitate, carefully avoiding any excess

Distilled water 550 vol.

⁴Pyridine favors the staining of fibrillary structures and at the same time increases the duration of this step. If an excess of pyridine is used the sections will have to remain longer in silver carbonate.

reduction (see step 7, below). Sections are placed on a slide and examined under lower power to see if fibers are stained. If so, a cover glass is placed over the section which is now examined with a high power dry objective ($\times 40$) to see if the desired fibers are stained. If they are, the whole slide is now placed in water and the section saved for final mounting or toning. As seen under the high dry objective, optimum conditions have been attained when the Purkinje cell dendrites are lightly and the fibers desired are darkly stained. Toning is optional but, in general, untoned sections are better for climbing fibers while toned are better for mossy fibers.

- (7) Tone in gold chloride solution, 1:600, until color of sections changes to purplish blue throughout.
- (8) If necessary, the toning may be intensified by warming sections in gold chloride at 45–50°C.
- (9) Fix in 5% sodium hyposulfite (sodium thiosulfate).
- (10) Dehydrate in alcohol.
- (11) Clear in carbol-xylene, to which a few drops of clear beechwood creosote have been added.
- (12) Mount in balsam.

With this technic cellular structures are lightly stained, favoring orientation, and all fibers are well demonstrated. When sections are toned, the background (cell bodies) becomes fainter.

PRECAUTIONS

All dishes (small 30 ml. dishes are recommended for silver solutions) must be cleaned with dilute nitric acid, tap water and then distilled water before use, particularly those used for silver solutions or those preceding immersion in silver. This must also be done with watch-glasses and manipulating rods, (glass rods thinned out in the tip and curved at right angles, 1 to 2 cm. from the tip, are recommended to manipulate sections). When coming back to pick up a section from the previous glass, the glass rod should be cleaned with distilled water (i.e., when coming back with the rod from carbonate to nitrate, from formalin to carbonate or from hypo to gold chloride).

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DIFFERENTIATION OF MYOFIBRILLAE, RETICULAR AND COLLAGENOUS FIBRILS IN VERTEBRATES

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ABSTRACT.—A procedure for the differentiation of the mesenchymal derivatives, myofibrillae, reticular and collagenous fibers is presented. Formol-Zenker fixation (5–12 hours) is followed by the washing, iodination, dehydration and paraffin embedding steps routine for that fixative with the following modifications. Zirkle's butyl alcohol series is used for dehydration and infiltration with paraffin as well as in the alcohol slide series. Embedding paraffin used is Parawax plus 8–10% bayberry wax. Tissue-exposed surface of paraffin block is soaked in water overnight before cutting serial sections at 3–5 μ . Sections are mounted using the dilute albumen method, and the slides, thoroughly dried at 37°C. overnight, are left at 60° for 10 minutes to melt the paraffin of the sections. Before staining, the sections are given a preliminary treatment with potassium permanganate and oxalic acid. For reticular staining a 10% silver nitrate bath is succeeded by an ammoniacal silver carbonate solution followed by reduction in 1% neutral formalin, toning in gold chloride and fixing in sodium thiosulphate. Myofibrillae, the sarcoplasmic limiting membrane and other sarcous elements are stained by Heidenhain's azocarmine solution, adult tissues at room temperature and fetal tissues at 50°C. Differentiation in phosphotungstic acid is followed by the staining of collagenous fibers. For adult tissue, light green SF (C.C.) is used and for fetal tissue, fast green FCF (C.C.). A discussion of the preparation of ammoniacal silver solutions is included. Both stock and used solutions of ammoniacal silver have been in use by the author for over a period of two years.

INTRODUCTION

In a recent investigation of the development of the muscle-tendon attachment in the rat (Long, 1947) a combination of staining methods for the study of muscle and connective tissue fibrils made possible a critical color determination among these tissue constituents. Although the use of silver, azocarmine, fast green or light green is well-known in the field of microscopic technic, their combination, in the method presented in this article, together with the specialized steps

in the technical procedure gave much better critical contrast than previously obtained.

The sarcolemma theory and the continuity theory are the two main opposing theories that have been expressed following observations on the relationships of myofibrillae and tendon fibrils. According to the continuity theory, myofibrillae are continuous with tendon attachment fibrillae. The sarcolemma theory holds that a sarcolemma intervenes between the muscle fiber with its sarcous elements and the connective tissue fibers of the tendon attachment region. The connective tissue stains most widely used in investigations of this problem have yielded results not sufficiently diacritical to resolve the contradictions in the two conflicting theories. Following the method outlined below, azocarmine stains the sarcous elements so that a pinkish sarcoplasmic limiting membrane is revealed at the ends of the muscle fibers. Myofibrillae (red) terminate on the inner aspect of this membrane and show no evidence of continuity with the argyrophilic (black) reticular fibers of the tendon attachment region. The attachment region lies between the end of the muscle fiber and its definitive tendon (Goss, 1944 and others.) The reticular fibers of this region are continuous proximally with endomysial and sarcolemmal argyrophilic fibers and distally with collagenous (green) fibers of the tendon.

This tinctorial differentiation of specific mesodermal derivatives has been studied in adult vertebrate tissues of the rat, monkey and man and in fetal rat material. It should prove of value whenever investigations are concerned with histogenetic and adult studies of mesodermal derivatives.

PROCEDURE

1. Fix in Formol-Zenker (95 ml. Zenker base + 5 ml. formalin on use) 5-12 hours depending on size of tissue.
2. Wash in running water overnight to 24 hours.
3. Dehydrate; treat with iodine for removal of Hg crystals. Infiltrate, and embed in paraffin. (Parawax with 8-10% bayberry wax).
4. Soak tissue-exposed surface of paraffin block in water overnight before cutting sections serially at 3 and 5 μ . Mount sections on chemically clean glass slides using the dilute albumen method (8-10 drops Mayer's Albumen in 30 ml. distilled water). Affix sections and thoroughly dry slides at 37°C. overnight. Immediately before proceeding with staining, leave slides 10 minutes or so at 60°C. to melt paraffin of sections. Slides are handled individually only in steps 16 and 29 to 35 inclusive.

5. Decerate in 2 changes xylene.
 6. Hydrate by graded steps including 10 minutes in 70% alcohol + iodine to insure complete removal of Hg crystals. Finally wash in running water for ten minutes.
 7. Rinse in distilled water.
 8. Treat in 0.25% aqueous potassium permanganate. 2 min.
 9. Wash in running water. 5 min.
 10. Rinse in distilled water.
 11. Treat in 5% aqueous oxalic acid. 2 min.
 12. Wash in running water. 5 min.
 13. Wash in distilled water, 3 changes, during ten minutes
 14. Treat in 10% aqueous silver nitrate, in dark. 12 + hr.
 15. Wash in 4 changes of distilled water
 16. Treat in ammoniacal silver carbonate solution in water bath at 40°-45°C. (covered staining jars). 25-30 min.
- Sections should be light or golden brown.

Preparation: Dissolve 10 g. silver nitrate in 20 ml. of distilled water. Add 200 ml. saturated aqueous solution of lithium carbonate very slowly (almost drop by drop). Allow precipitate to settle. Decant and wash precipitate 5 times with distilled water. Dissolve precipitate with ammonium hydroxide very slowly, drop by drop, agitating continuously. Leave few grains of precipitate and add distilled water to 200 ml. Filter and heat uncovered at 50°C. for 30 minutes. Filter again before use. Solution should be at pH 10+. Solutions of effective pH may be used repeatedly. Heat to 50°C. and filter before re-using. Such solutions have been kept in brown glass-stoppered bottles and used over a period of two years.

17. Plunge each slide individually into a dish containing 500 ml. distilled water plus 4 drops of ammonium hydroxide.
 18. Immediately transfer to 1% neutral formalin. 10-20 min.
 19. Wash in running water. 10 min.
 20. Rinse in distilled water
 21. Tone in 1/500 aqueous gold chloride. 15-30 min.
 22. Rinse in distilled water
 23. Fix in 5% aqueous sodium thiosulphate. 2 min.
 24. Wash in running water. 10+ min.
 25. Rinse in distilled water
 26. Stain in azocarmine:
 - Adult tissues at room temperature. 15-30 min.
 - Fetal tissues at 50°C. 30 min.
- Complete cooling not essential before next step.

Preparation: Dissolve 0.1 g. azocarmine¹ in 100 ml. distilled water by bringing to a boil. Cool to room temperature. Filter immediately. Add 1.0 ml. glacial acetic acid.

27. Rinse in distilled water.
28. Treat in 5% aqueous phosphotungstic acid.....3-6 hr.
Differentiate until collagen fibers are decolorized. Change solution when it reddens.
29. Rinse in distilled water.
30. Stain in one of the following.....3 min.
Adult tissues—1% light green SFY² (C.C.) in 1% aqueous glacial acetic acid.
Fetal tissues—1% fast green FCF³ (C.C.) in 1% aqueous glacial acetic acid.
31. Rinse in distilled water.
32. Rinse in 1% aqueous glacial acetic acid. Differentiate if necessary.
33. Rinse in distilled water.
34. Rinse in 95% alcohol.
35. Complete dehydration in the following solution: 95 ml. 95% ethyl alcohol + 5 ml. normal butyl alcohol.
36. Clear in: 95 ml. xylene + 5 ml. normal butyl alcohol.
37. Pass into xylene, 3 changes, 5 minutes each.
38. Mount in damar or Clarite.

DISCUSSION

Fixation with 10% formalin allows adequate staining of reticular fibers in this procedure but it was found that the use of formal-Zenker was followed by more effective staining with azocarmine and the green stains, particularly in the series of fetal tissues. Following formal-Zenker fixation of fetal rat tissues, definitive collagenous fibers of the Achilles tendon take the fast green stain and therefore presumably assume the properties of collagen during the 18-day fetal stage. Following 10% formalin fixation the Achilles tendon fibers do not appear collagenated until after birth.

Zirkle's butyl alcohol series (Zirkle, 1930) is recommended for dehydration and infiltration with paraffin as well as in the alcohol

¹Azocarmine Grüber and Co. Leipzig.

Azocarmine G (C.C.) also was used effectively, dye content and certification number not known at present writing.

²Light Green S F (C.C.) dye content 70%. Coleman and Bell Co.

³Fast Green FCF (C.C.). First batch used, dye content 95%, certification No. NGf-5; second batch used, dye content 91%, certification No. NGf-4. Both batches products of the National Aniline and Chemical Co., Inc.

slide series. Connective tissues notoriously harden during dehydration and clearing procedures. The use of butyl alcohol in the dehydration series and as a clearing agent permits more uniform serial cutting of sections than when an ethyl alcohol dehydration series and other common clearing agents are used in the processing of the tissues. The infiltration of water through the exposed surface of the paraffin block as indicated in step 4 also tempers the hardness of the connective tissues.

As in all staining technics involving the use of ammoniacal silver solutions special care must be exercised in the preparation of the solution and in the impregnation and reduction steps of the procedure. Kubie and Davidson (1928), in their outstanding paper on the chemistry of the ammoniacal silver solutions, recommended the use of equimolar solutions for more accurate and reproducible results. Following a comparison of the three types of ammoniacal silver solutions, i.e. ammoniacal silver nitrate, ammoniacal silver hydroxide and ammoniacal silver carbonate, they concluded that the carbonate solution is a more effective impregnating agent because of its intermediate type of stability and its reserve alkalinity due to the buffering action of its constituent carbonate salts. During the reduction of silver solutions by formaldehyde, formic and nitric acids are produced which block the further reduction of ammoniacal silver solutions, since silver cannot be reduced in an acid solution. During reduction of ammoniacal silver carbonate by formaldehyde the buffering action of the carbonates offsets the action of the acids and the reduction process goes closer to completion. Foot (1929) seconded the recommendations of Kubie and Davidson and suggested the value of adding a buffer as well to the formalin developer. Silver (1942) buffered both the ammoniacal silver carbonate solution and the formaldehyde reducing solution to definite pH points. In one series of experiments specifically designed for the rendition of reticulum of frog liver, Silver concluded that the silver staining of reticular fibers occurs most completely at pH 10. Solutions of pH 8 and pH 12 yielded erratic and faint staining of reticular fibers.

In the preparation of the ammoniacal silver carbonate solutions used in the procedure under discussion (see step 15) solutions were prepared in the traditional del Rio-Hortega manner (not using equimolar solutions). In an attempt to ascertain as accurately as possible the most effective pH for reticulum staining, ammoniacal silver carbonate solutions of pH 9.88, 10.35, 10.4, 11.12, 11.2, 11.5 and 11.8 were prepared. These solutions were obtained by adding more NH_4OH at the crucial time in the preparation of the solution when

the end point of precipitate-solution is reached. The pH determinations were made on a Cambridge glass electrode⁴. Impregnation of sections with the first three solutions resulted in complete and precise staining of reticular fibers, a result which coincides with Silver's conclusion. All solutions in the pH 11 range gave spotty and capricious reticular staining.

The stock impregnation solutions of pH 10.35 and 10.4 have been used by the author for staining of reticulum over a period of two years. Used solutions have been re-used as many as six times. Solutions are heated and filtered before re-using. Some slight precipitation occurs on standing but no noticeable diminution of the effectiveness of the staining qualities is evident. This statement concerning the use of both old stock and used ammoniacal silver solutions is somewhat contrary to established technical procedure. Laidlaw (1929) indicates that the stock solution keeps for many months. Most investigators specify that the solution must be made fresh or used at the most within a period of two days (Lillie, 1946). Since the preparation of the solution is time consuming and detailed, its re-use as well as its availability as a stock solution is of decided advantage. Recently the pH of the two-year old pH 10.4 solution was redetermined. The pH had dropped to pH 9.65 but the solution still yielded effective reticular staining of fetal rat tissues. These limited trials for determination of the pH range for effective reticular staining indicate that the range is at least from pH 9.65 to pH 10.4. Adult and fetal reticulum respond equally as well to impregnation solutions within the pH range. Studies for the determination of the full pH range effective for reticulum staining are in progress.

The dilute ammonia rinse (step 16) seems to remove precipitated Ag and AgOH from the slides and thus permits study of the argyrophilic reticulum uncomplicated by silver precipitation in the sections. Buffering of the reducing solution in this procedure is probably attained by the neutralization of the formaldehyde stock solution with lithium carbonate. The writer, however, believes that equimolar solutions should be used in the preparation of both the impregnation and reduction solutions as suggested by Kubie and Davidson, Foot and Silver. These solutions buffered to the pH effective for the staining of reticulum should give exact and reproducible results in all studies embodying reticulum in the hands of inexperienced as well as experienced workers in fields where ammoniacal silver solutions are indicated.

⁴pH determinations were made by Dr. S. R. Tipton of the Department of Physiology of the Medical College of Alabama.

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IMPREGNATION OF OLIGODENDROGLIA IN NERVOUS TISSUE KEPT IN FORMALIN FOR MANY YEARS

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ABSTRACT.—A method for impregnating oligodendroglia in nervous tissue (monkey) fixed and preserved in formalin for many years is described. This tissue is reconditioned by placing 12 to 30 μ frozen sections of it in concentrated ammonia (sp. gr. 0.90) and by washing them slowly for 24 hours with a 1 mm. stream of water. The fluid is then poured off the sections; the jar is refilled with concentrated ammonia; and washing is repeated for another 24 hours. The sections are then plunged into concentrated ammonia for 7 minutes.

After treatment in ammonia, the sections are incubated for one hour at 38°C. in Globus' 5% hydrobromic acid solution. They are washed again, in distilled water, and then impregnated in a "medium" strength ammoniacal silver carbonate solution (5 ml. of 10% AgNO_3 added to 15 ml. of 5% Na_2CO_3). The precipitate is dissolved in concentrated ammonia and diluted to 50 ml. with distilled water). Impregnation is followed by reduction in 1% formalin without agitation; fixation in 5% $\text{Na}_2\text{S}_2\text{O}_3$; dehydration, and mounting in clarite.

Typical oligodendroglia (Fig. 1) were made visible by use of the method outlined in this paper.

The method described by Hortega (1921) for the impregnation of oligodendroglia in his classical description of those supporting cells in the central nervous system, called for fixation in Cajal's formalin-ammonium-bromide (known as FAB) for 12 to 48 hours. Later Hortega is said to have extended the time allowed for fixation to 72 hours (Buño, 1947). Buño, who worked in Hortega's laboratory in South America, reports that Hortega considered that prolongation of this time of fixation in either FAB or formalin rendered impregnation of oligodendroglia difficult. If it were necessary, however, to use nervous tissue fixed for longer periods of time (three months was considered the upper limit) sodium bisulphite was some times used as a "rejuvenating" agent and in the method a "strong" ammoniacal silver carbonate solution was substituted for a "weak" solution of that compound.

Penfield's first modification (1924) of Hortega's technic for impregnation of oligodendroglia shortened the time of fixation in Cajal's formalin-ammonium-bromide from a range of 12 to 72 hours to that

of 2 to 12 hours. Globus' (1927) method of impregnating the neuroglial elements in nervous tissue "which had been kept in formaldehyde solution for months or even years" did not bring out the cytoplasm of the oligodendroglia. Rather, only the nuclei were evident as darkly stained spherical bodies. A year later Penfield (1928) published a second modification for impregnation of oligodendroglia and neuroglia. Although he says that the tissue may be hardened "in 10% formalin (or formalin-ammonium-bromide) for an indefinite period", he limits good results for oligodendroglia to approximately a week's fixation.

The impregnation of oligodendroglia in material which has been preserved in formalin for many years has proven to be refractory to the method of Globus and to that of Penfield's "second modification" as well. Since formalin is in general use as a fixative and preservative of the central nervous system, a method allowing impregnation of "the most numerous of the supporting cells in the nervous system" (see p. 491, Penfield and Cone, 1937) might prove valuable.

The method which is described in this paper is the preliminary step in a study of oligodendroglia in a series of young monkeys which have been preserved in formalin for many years.

MATERIAL

The material used in this study was taken from the spinal cord of a 10-week old monkey (*Macaca mulatta*). This animal was perfused by 10% formalin under anaesthesia on January 6, 1934. For 13 years the exposed spinal cord remained *in situ* in 10% formalin.

METHOD

The method used falls naturally into two divisions, first that of reconditioning the tissue and second, that of a minimal but what seems to be crucial modification of Hortege's ammoniacal silver carbonate technic for the impregnation of oligodendroglia.

A. Reconditioning.

1. Cut frozen sections 12 to 30 μ in thickness.
2. Put 12 to 18 sections in a 100 ml. jar; fill with concentrated ammonia (sp. gr. 0.90) and cover with gauze mesh drawn taut and secured by white thread.
3. Wash for 24 hours with water (distilled or tap) flowing slowly enough barely to stir the sections. This minimal but necessary agitation of the sections was made possible by passing the stream of water through a glass tube drawn to a 1 mm. bore at the end which is inserted between the mesh of the gauze. Contamination by metals, such as rust, must be avoided.

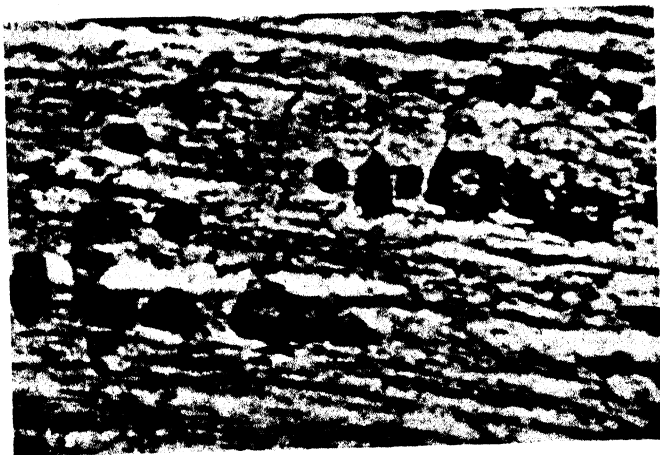


FIG. 1.—Untouched photograph showing the interfascicular arrangement of several typical oligodendroglia in a sagittal section of the spinal cord (T_3) of a 10 week old monkey (*Macaca mulatta*). This spinal cord was kept in 10% formalin for 13 years. $\times 820$. Observed with 8 mm. apochromatic Spencer lens and $\times 20$ compensating ocular.



Fig. 2.—Untouched photomicrograph showing several typical oligodendroglia with clear nuclei and dark cytoplasm. Fine prolongations are also seen. $\times 400$. Observed with a 8 mm. apochromatic objective and a $12.5 \times$ compensating ocular.

4. Pour off the water; refill the 100 ml. jar with concentrated ammonia (sp. gr. 0.90) and repeat the washing as outlined above for another 24 hours.¹
5. Transfer the sections with a bent glass rod to concentrated ammonia (sp. gr. 0.90) and allow to remain for 7 minutes. (Beginning with this procedure all glassware should be cleaned with concentrated nitric acid and no metal should be allowed to touch the section until its final washing.)

B. Modification of Hortega's method for impregnation of oligodendroglia.

1. The sections are transferred directly from the concentrated ammonia (sp. gr. 0.90) to Globus' 5% hydrobromic acid solution (5 ml. 40% hydrobromic acid plus 95 ml. distilled water). Heat to 38°C. in an incubator for 1 hour.
2. Wash the sections thoroughly in four changes of distilled water. Leave in each change for four minutes. (Sections are stained at room temperature.)
3. Place the sections in a small dish containing about 15 ml. of "medium" strength ammoniacal silver carbonate solution, prepared in the following way: To 5 ml. of 10% AgNO_3 add 15 ml. of 5% Na_2CO_3 . Dissolve the precipitate by adding, drop by drop, concentrated ammonia, and then dilute to 50 ml. with distilled water. No odor of ammonia should be present after the precipitate has been dissolved.

The sections must remain in the ammoniacal silver carbonate solution until, following reduction in 1% formalin, oligodendroglia as such are visible when the section is examined under the microscope. The time interval for impregnation with silver varies from 10 to 75 minutes, but was generally found to be between 30 and 45 minutes.

4. Remove a section every 5 to 10 minutes and wash for 10 to 15 seconds in distilled water. Agitate in the water.

¹Washing the sections out of concentrated ammonia (sp. gr. 0.90) for two days with a change after the first day was found to give excellent results after many other concentrations of ammonia, followed by washing in running water for various times, were tried. Washing the sections in running water or soaking them in water for four days was an unsuccessful treatment, as was soaking them in a 1, 5, 10, 15, or 25% solution of ammonium hydroxide (as well as a concentrated solution) followed by washing out the sections from each of the above solutions for one to ten days. Only fair results were obtained when the sections were placed in 25% ammonia and then, without preliminary soaking in this solution, washed for five to ten days or when the sections were placed in concentrated ammonium hydroxide and washed out for one day. The repetition of this last procedure for the second day gave excellent results and therefore no more efforts at reconditioning the tissue were tried.

5. Reduce the section by sliding it very gently into 1% formalin solution. Do not agitate. Reduction of the impregnated silver will be complete in thirty seconds. The sections may remain in this solution for a longer period of time without deleterious effects.
6. Wash the section briefly in distilled water and examine under the microscope. (Impregnation is complete and reduction is ideal when the cytoplasm and the prolongations of the oligodendroglia are visible. When this occurs, the remaining sections should be taken out of the impregnating solution, washed in water as above, and reduced one by one).
7. When the oligodendroglia are visible, return the section to distilled water, and wash by continual agitation until all excess silver on the surface of the section is removed. After the excess silver is removed sections may remain as long as four days in water with impunity.
8. Fix in 5% $\text{Na}_2\text{S}_2\text{O}_3$ for 2 minutes.
9. Wash briefly in distilled water (about 30 seconds).
10. Dehydrate the sections in 95% alcohol for one minute. Longer dehydration distorts the oligodendroglia.
11. Clear the sections in creosote for one minute; blot with onion skin paper or thin filter paper, and mount in clarite. Creosote causes less distortion than xylene and counteracts the shrinkage produced by 95% alcohol.

In Hortega's laboratory the dehydration is carried out on a glass slide. The section is placed on the slide and covered with 95% alcohol. Two changes of alcohol are sufficient for partial dehydration. A few drops of creosote are placed on the section. When clearing is complete the section is blotted with onion skin paper, or thin filter paper, and mounted in Canada balsam. (Buño, 1947).

DISCUSSION

In the method outlined for impregnating oligodendroglia in nervous tissue which has lain for 13 years in 10% formalin, ammonia was used in two ways, (1) for reconditioning the tissue and (2) for forming ammonium bromide within that tissue. Although the use of ammonia for reconditioning nervous tissue is not original (Globus first used it in 1927) the method of its use is. The slow washing of sections in great excess of concentrated ammonia seems to liberate the formalin and formalin-ammonia complexes (one of which may be hexamethylene tetramine) more readily from the tissue itself as well as from the fluid which surrounds it.

The second use of concentrated ammonia (the sections are soaked in this solution for 7 minutes, reconditioning, step 5) immediately before bromination, facilitates the formation of ammonium bromide when the sections are plunged into 5% hydrobromic acid. Ammonium bromide must be formed within the tissue itself before impregnation when ammonium bromide is not used as a part of the original fixation (Globus, 1927).

Important as these two uses of concentrated ammonia are, no free ammonia should be left in the tissue at the time it is placed in the impregnating solution. (See also Hortega, 1928; and Penfield, 1928). Ammonia disturbs the delicate balance of the ammoniacal silver carbonate solution.

Although Hortega used a "strong" ammoniacal silver carbonate solution for impregnating oligodendroglia in "old" tissue (Buño, 1947) and Penfield (1928) a "weak" solution of that compound, in our experience a "medium" strength solution gives a satisfactory impregnation of oligodendroglia in tissue kept in formalin for many years. In addition, the time allowed for impregnation is increased beyond that recommended by previous workers.

At the suggestion of Buño (1947) of the University of Montevideo, the writer tried Hortega's (1921) original method of allowing the reduction of the impregnated section to take place without agitation in 1% formalin. In the writer's experience agitation of the section in the reducing fluid increased the number of microglia impregnated and decreased the number of oligodendroglia. This result was contrary both to Hortega's later direction for "stirring constantly" (1928) and to Penfield's (1924; 1928) finding that agitation gave the most fruitful results.

The writer is very glad to express his indebtedness to Dr. Marion Hines for suggesting the problem and for assistance in preparation of the manuscript.

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A SIMPLIFIED AND CONVENIENT METHOD FOR THE DOUBLE-EMBEDDING OF TISSUES

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ABSTRACT.—A method for embedding tissues with a celloidin-paraffin combination is presented. The essential features of the process depend upon (1) a thorough infiltration of the specimen with celloidin of low concentration, and (2) the subsequent impregnation of both the specimen and the celloidin with paraffin.

The methods for sectioning, and the removal of the embedding agent are given.

The chief advantages of this method are: the preservation of all of the advantages of celloidin embedding but with a great saving of time, and greater convenience of storage; the cutting of thin sections (2μ for many types of tissues); it is useful for embedding specimens for which neither pure paraffin nor pure celloidin are entirely satisfactory, i.e. those containing tissues differing in density.

Various embedding substances have been employed in the preparation of tissues for sectioning. The procedures required in the use of each embedding agent possess certain disadvantages which are tolerated only because of some particular feature or features intrinsic to the specific substance selected. Thus, in the widely used paraffin method, the disadvantages of tissue shrinkage and distortion, and compression against the knife during sectioning, (the latter partly controlled by the addition of beeswax or bayberry wax), are liabilities of this technic but they are disregarded in favor of the simplicity and speed of the process. Conversely, celloidin does not enjoy such common usage as an embedding material because of the prolonged time consumed in the process even though tissues are practically free from shrinkage and distortion, advantages decidedly in its favor. Then, too, there is the added disadvantage of storage since the celloidin blocks or sheets must remain continually submerged in alcohol in suitable containers until they are used.

Other procedures, such as the different varieties of the paraffin-sugar-celloidin method, and the celloidin-paraffin combinations heretofore in use, have not been so frequently employed either because of the complexity of the procedures for all but experts skilled in these technics or because the field of applicability is somewhat restricted according to the character of the tissue to be embedded. While the

freezing technic is extremely useful in preparing representative sections from a variety of tissues for rapid diagnosis or for a study of tissues unaltered by fixatives, its limitations are well known when either serial sections or consistently thin sections (less than 10μ) are desired. The use of gelatin as an adjunct for cutting frozen sections detracts from the value of the plain freezing method in one respect by requiring a fixative, yet it does permit the cutting of relatively thinner frozen sections. At the same time, the complexity of the process is increased thus limiting its use to certain special phases of study.

A superior embedding agent should embrace, among other things, the characteristics of (1) simple, easy and fairly rapid preparation; (2) consistent results even in the hands of the untrained individual; (3) minimal tissue distortion due to embedding materials and procedures; (4) elimination of compression artifacts during the cutting process; (5) adaptability to various tissues differing in density; (6) the ability to be cut in thin serial sections (as low as 2μ for many types of tissues); (7) convenient storage of the embedded blocks.

Credit is due to the late George S. de Renyi for many of the refinements of the following double-embedding technic and for personally introducing the writer to it some years ago.

METHOD FOR DOUBLE-EMBEDDING

1. Fix the specimen with the fixative of choice or stain in toto, then thoroughly dehydrate. In order to minimize shrinkage caused by dehydration, start with 20% alcohol and advance by 5% stages (6 to 8 hours in each) to 95% alcohol at which concentration 2 changes (24 hours each) are necessary.

2. Two changes of absolute alcohol—12 hours each.

3. Absolute alcohol and ether (equal parts) 6 to 12 hours depending upon the size of the specimen.

4. Celloidin, 2%—12 to 24 hours, in covered stender dish.

5. Celloidin, 4%—12 to 24 hours, in covered stender dish.

6. Celloidin, 6%—48 to 96 hours, in covered stender dish.

7. Remove the specimen and place it in an open, paraffin-lined stender dish (suitable size) containing 6% celloidin sufficient in amount to cover generously the specimen. (Bubbles which may occur in the celloidin are of no importance and may be ignored.)

8. Place the paraffin-lined dish with contents in a larger covered vessel containing only enough chloroform to cover the bottom. (The specimen must be oriented at this time because the chloroform vapors will harden the celloidin within several hours. The surface to be sectioned first should face the bottom of the paraffin-lined dish.)

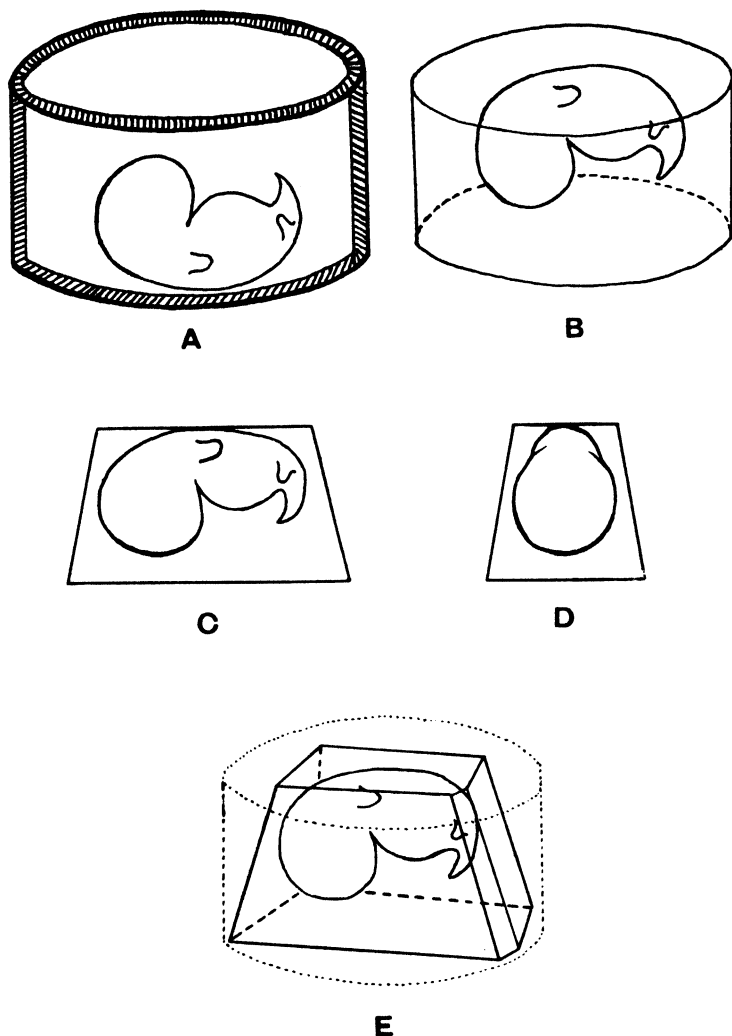


FIG. 1.—A. Paraffin-lined stender dish containing specimen in 6% celloidin.
 B. Firm celloidin mass after removal from the paraffin-lined dish; inverted and ready for trimming.
 C. Side view of trimmed block.
 D. End view of trimmed block.
 E. Perspective of trimmed block in its definitive form with bevelled leading corner, shown in relation to the outline of the original cylindrically-shaped mass of celloidin.

Do not remove from the chloroform vapors until the celloidin is moderately firm (not hard) throughout.

9. Remove the celloidin block from the paraffin-lined dish (teasing needle or similar instrument passed around the paraffin layer will loosen it), turn upside down and quickly trim away the excess celloidin in such a way that the surface to be sectioned first may be identified readily. A suggested method is schematically shown in Fig. 1. Do not let the block dry out during the trimming process.

10. Clear the block in 3 changes of generous amounts of benzene (benzol). An amount 4 or 5 times the volume of the block is sufficient for each of the following:

- (a) Benzene No. 1—12 to 18 hours. Benzene should be discarded after use because of the chloroform in it.
- (b) Benzene No. 2—6 to 12 hours. This may be re-used as benzene No. 1.
- (c) Benzene No. 3—6 to 12 hours or until cleared. This may be re-used as benzene No. 2. Benzene No. 3 must be kept fresh.

11. Transfer the cleared block to a thoroughly saturated mixture of paraffin (47°–50°C. m.p.) in benzene at a temperature of 37°C. or in a warm room. Allow the block to remain for 24 hours. Cover the container to retard evaporation of benzene.

12. The block is then impregnated with melted paraffin as follows:

- (a) Paraffin No. 1 (47°–50°C. m.p.)—12 to 24 hours.
- (b) Paraffin No. 2 (53°–55°C. m.p.)—12 to 24 hours.
- (c) Paraffin No. 3 (53°–55°C. m.p.)—12 to 24 hours.

13. Place the block on a piece of stiff paper or thin cardboard with the surface to be sectioned facing the paper. Submerge the paper with the block on it in cold water for a long enough time to permit thorough hardening of the paraffin. Remove the block from the paper, label and store in a relatively cool, dry place.

SECTIONING

All roughened surfaces of the embedded block should be trimmed smooth. The corner designated to lead against the knife should be bevelled (Fig. 1). This will not only facilitate the cutting process but also impart to all sections a distinguishing characteristic useful for orientation.

The base of the block is securely fastened with paraffin to a block holder which is then properly adjusted and clamped in a sliding

microtome¹. The upper surface of the tilted, slanting knife should be wiped clean with ether, then smeared with a thin coating of albumen (Mayer's), allowing a few minutes for drying. Since the double-embedded sections are cut with a knife flooded with 30% alcohol, the albumen on the knife materially aids in the retention of the alcohol.

After one section has been cut, it is slid towards the end of the knife by pulling it with a teasing needle or pushing it with a medium sized camel's hair brush wet with 30% alcohol. Several consecutive sections of average size may be collected in a row in this manner. The sections are then removed from the knife. With the camel's hair brush soaked with 30% alcohol, the knife is flooded above the sections and, gently pushing them if necessary, they are floated onto a mica spatula placed against the under side of the knife edge. Large sections may be removed individually in a like manner or they may be lifted from the knife with forceps. If, for some reason such as the improper tilt of the knife, the section curls when cut, it should be flattened on the knife before removal. The top surface of the block should be wiped dry with the finger before the next cutting stroke.

If serial sections are desired, those that have been collected and floated in order onto the mica spatula are transferred directly to an albumenized slide. The excess 30% alcohol is removed by draining. The sections are washed 2 or 3 times with distilled water by alternately re-floating on the slide and draining the excess. An eye dropper pipette is useful for this. These sections are then dried either in a drying oven or not, according to personal election. Placing the slides on a warming tray is helpful in flattening out the sections on the slide.

If serial sections are not desired, they are transferred from the mica spatula to a shallow dish of distilled water on which the sections float. No harm occurs to the material if allowed to remain thus for long periods of time (months). The sections may be subsequently drawn up onto albumenized slides.

REMOVAL OF THE EMBEDDING AGENT

Double-embedded sections must have the paraffin removed first. The following steps are for handling sections already mounted on slides:

¹Sectioning must be done with a wet knife (sliding microtome). Cutting with a dry knife (rotary microtome) is not possible since the knife does not pass easily through the block because of too much "drag" as it goes through the celloidin. The sections, which become much compressed, tend to stick against the blade. Satisfactory sections and hence, ribbons are impossible.

1. Xylene—2 to 5 minutes.
2. Absolute alcohol—2 to 3 minutes.
3. Absolute alcohol and ether (equal parts)—10 to 20 minutes.
4. Absolute alcohol—2 to 3 minutes.
5. Alcohol, 95%—2 to 3 minutes.
6. Hydrate through graded alcohols to distilled water.

From distilled water, the sections may be stained according to any of the standard methods including the silver-on-the-slide technics. For alcoholic stains, the sections must be brought into distilled water first, then back through the alcohols until the desired level of concentration has been reached.

DISCUSSION

The celloidin-paraffin technic that has been described produces an embedding agent to which each component has contributed valuable attributes. Such a final product differs materially from the one obtained when the usually described double-embedding method (Lee, 1937, p. 104) is followed. For the latter process, the celloidin infiltration must progress to the maximum (16%) stage; paraffin is used only for hardening the final celloidin block. Not only does this prolong further an already lengthy technic but it is doubtful whether sufficient advantage is gained to warrant the addition. The effectiveness of the celloidin-paraffin combination herein presented depends upon a thorough infiltration of the specimen with celloidin of low concentration² only, and the subsequent impregnation of the specimen and celloidin with paraffin, the apparent behavior of which is to fill the interstices of the partially concentrated celloidin.

The advantages offered by this simplified and convenient method for double-embedding may be summarized as follows:

1. The time required for embedding has been reduced to a matter of days, an item of considerable importance when compared to several weeks—perhaps even months—needed for pure celloidin embedding. Paraffin embedding is more rapid than either of these two methods.

2. Tissue relationships remain undistorted in a manner comparable to pure celloidin embedding. This is not entirely true when paraffin alone is used as the embedding agent.

3. Cutting of very thin sections (10–2 μ) serially is possible for many tissues. Celloidin-paraffin embedding is not recommended when sections are to be cut thicker than 15 μ .

²The hot celloidin method [Wetmore (1932), Walls (1932)] could be employed until the 6% celloidin stage only has been reached; this can be followed quite satisfactorily by the paraffin procedures for double-embedding.

4. Compression artifacts are negligible at the time of cutting the sections but resemble pure celloidin embedded material in this respect. Compression artifacts are well known in critical paraffin sections especially when thin.

5. In addition to its general usefulness, this double-embedding technic finds special application for the embedding of specimens for which neither pure paraffin nor pure celloidin is entirely suitable, i.e. those consisting of tissues differing widely in density.

6. Embedded blocks may be stored easily and conveniently. They will not "flow" like paraffin in warm weather. There is no necessity for them to be submerged in alcohol, thus avoiding the use of separate storage containers.

7. The process is simple and easy to execute. If the prescribed steps are adhered to, consistent results are the rule even when performed by an individual untrained in this technic.

8. The methods for sectioning and removal of the embedding agent have been given.

REFERENCES

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- WALLS, G. L. 1932. The hot celloidin technic for animal tissues. *Stain Techn.*, 7, 135-45.
- WETMORE, R. H. 1932. The use of celloidin in botanical technic. *Stain. Techn.*, 7, 37-62.



LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

PIJPER, A. A new way of applying immersion oil. *J. Path. & Bact.*, **59**, 486. 1947.

A 30 ml. Schuster bottle, provided with a long and narrow spout arising from the neck of the bottle and tilted upwards at an angle of 45°, is a good container for doling out immersion oil in exact drop sizes at precisely the site desired. The oil is thus protected from contamination and evaporation. The wall of the flask opposite the base of the spout has a corked opening for replenishing the oil.—S. H. Hutner.

PHOTOMICROGRAPHY

KINGMA BOLTJES, T. Y. Some remarks on microphotography. *Antonie Van Leeuwenhoek*, **12**, 232-42. 1947.

Consideration is given to various refinements in technic in connection with critical photomicroscopy. Special attention is given to the use of ultraviolet light of 365 mμ obtained from a mercury vapor lamp (e.g. Philips HP 300), with the proper filter. Although this light is not far from the visible violet, it makes no impression on the eye and accordingly cannot be used for focusing. The writer therefore focuses with the use of a filter giving light of 436 mμ, and then determines by means of six or eight test photographs how much the microscope tube must be raised to obtain sharp focus with light of 365 mμ; this factor once learned, it can then be applied to later focusing with the same instrument, provided care is taken to make all fine adjustments in the same direction of the screw, to avoid backlash. The writer concludes that the greater resolving power of the ultraviolet light is worth taking advantage of, but that other refinements such as use of apochromatic objectives are of less value; this latter observation derives from the fact that apochromats have no theoretical advantage over achromats when monochromatic light is used.—H. J. Conn.

DYES AND THEIR BIOLOGICAL USES

KASHA, M., and POWELL, R. E. On the correlation of the spectroscopic and thermal energy differences between the fluorescence and phosphorescence levels of dye molecules. *J. Amer. Chem. Soc.*, **69**, 2909-10. 1947.

An interpretation of the fluorescent and phosphorescent properties of polyatomic molecules on the basis of potential curve crossing suggests a limitation in the validity of the mechanism of luminescence of dyes proposed by Jablonski. According to Jablonski, the spectroscopic energy difference between the zero-point energy levels of the fluorescent (unstable) and phosphorescent (metastable) states of a dye should be equal to the heat of activation required for lifting molecules from the phosphorescent to the fluorescent state. However, according to the mechanism of excitation of the phosphorescent state based on potential curve crossing, the two experimental energy quantities may differ considerably in magnitude, if the point of crossing is not at the lowest point of the upper curve. According to this picture, the heat of activation should equal or exceed the spectroscopic difference between the zero-point levels of the fluorescent and phosphorescent states.—R. T. Whittenberger.

NEWTON, W. An indicator agar for the determination of the relative concentration of ascorbic acid in potato tuber tissue. *Canad. J. Res.*, 25, 242-5. 1947.

To determine by a simple means the concentration of ascorbic acid in potato tuber tissue as an index of virus disease infection, the following indicator agar method was developed: heat 2 g. agar, 75 ml. water, 10 ml. 10% KI, and 5 ml. of a 1% solution of soluble starch; cool to about 60°C.; add 5 ml. glacial acetic acid and 5 ml. 0.02N KIO₃; pour immediately into Petri dishes, 10 ml. per 9 cm. dish, or proportionately more for larger dishes; have depth of agar uniform; store in refrigerator until used; place filter paper discs saturated with ascorbic acid solutions on agar surface. If depth of agar is constant and loss of iodine negligible in preparing the medium, diameters of halos around discs are the same after 24 hr. at 5°C. when discs are saturated with standard ascorbic acid solution. Remove tissue cylinders from potato and cut slices 5 mm. thick; place on agar and keep in refrigerator; observe size of halos after 24 hr. Discs from tubers affected with mosaic and leaf roll decolorize a greater area than discs from normal tubers of same variety; upper surfaces of discs of virus-infected plants tend to remain white but those from healthy plants become blue-black. This method serves as a rapid method of determining approximate concentrations of ascorbic acid in miscellaneous fluids.—H. P. Riley.

ANIMAL MICROTECHNIC

ASH, J. E. Technical methods in use at the Army Institute of Pathology. *J. Techn. Methods*, 26, 1-59. 1946.

This is a useful manual of histological and related methods used in the Army Institute of Pathology. The paper is divided into the following sections: General procedures; Routine general stains; Connective tissue stains; Methods for staining nerve tissues; Methods for staining micro-organisms; Method for staining blood; Methods for staining cell products; Museum methods. It has been prepared by the author in response to numerous requests for information; and the object of the publication is to summarize the procedures in a form in which they may be readily available.—H. J. Conn.

HOVANITZ, WILLIAM. An electron microscope study of isolated chromosomes. *Genetics*, 32, 500-4. 1947.

A study was made of isolated chromosomes from "resting" nuclei of chicken red blood cells. The cells were washed in 0.14 M NaCl and physically broken up in a high speed blender. The chromosomes were then centrifuged out and the cytoplasm washed away, leaving a large quantity of chromosome threads as a residue. The chromosomes were fixed with such standard fixatives as osmium tetroxide, acetic-alcohol and chromic acid, transferred to distilled water and a drop of suspension dried upon a collodion electron microscope screen. Micrographs of the chromosomes were obtained directly or they were shadow cast, which allows for the determination of the third dimension of electron microscope views. A definite spiral structure was apparent in the chromosomes, appendages were seen on chromosomes, but no definite chromomeres were apparent.—John Einset.

LENDRUM, A. C. The phloxine-tartrazine method as a general histological stain and for the demonstration of inclusion bodies. *J. Path. & Bact.*, 59, 389-404. 1947.

This technic is offered as a substitute for Masson's erythrosin-saffron method, with the advantages of affording a brilliant demonstration of certain inclusion bodies, and of making use of a stable phloxine solution.

The recommended procedure is as follows: Stain with Mayer's hemalum as usual. Iron alum hematoxylin may also be used. Differentiate and blue. Stain with 0.5% phloxine in 0.5% CaCl₂ for 30 min. Rinse briefly in water, drain, and replace with a saturated solution of tartrazine NS (Imperial Chemical Industries) in cellosolve, using a dropping bottle to control the differentiation. The yellow tartrazine acts to replace the red phloxine from collagen. As tartrazine is very soluble in water, overstain slightly before dehydration. Rinse in 60% and 95% alcohol, absolute alcohol, and xylene.

Kurloff bodies in guinea pig lung are well shown. There is retention of phloxine in the inclusion bodies of a number of virus-containing tissues. Fixatives containing HgCl_2 give good results. The method is illustrated with color plates. The stain is also illustrated by a drawing in color in an adjoining paper (Cappell, D. F., and McFarlane, Marjory N., *J. Path. & Bact.*, **59**, 358-398. 1947).—*S. H. Hutner.*

MICROÖRGANISMS

CONN, H. J., and ELROD, R. P. Concerning flagellation and motility. *J. Bact.*, **54**, 681-7. 1947.

Beautiful electron microscope pictures of bacterial flagella were obtained as follows: Grow bacteria for about 16 hr. on fresh lima bean agar slopes or plates; touch a clean microscope slide to the growth; add a drop of distilled water to the adhering bacteria or transfer a loop of material to a drop of water on the slide; let stand 30 min.; flood slide with distilled water; rotate gently a few times and pour off water; dry in vertical position; shadowcast with 8-10 Å of gold at an angle of 15°; run a solution of 0.5% collodion in amyl acetate over the slide; dry in vertical position; float off the film and mount it on the screen. This technic, which usually results in shadowcast replicas on the screen, was found to incorporate the organisms as well as the gold in the collodion and to show most of the bacteria with intact flagella.—*Virgene Kavanagh.*

CORPER, H. J., and COHN, M. L. Contrast coloring of media by dyes in growing tubercle bacilli. *Amer. J. Clin. Path.*, **16**, 621-33. 1946.

A number of vital, azo, azine, triphenylmethane and other dyes were added to egg yolk glycerol medium to determine which would impart a color to the medium in sharp contrast to that of colonies of tubercle bacilli. In addition it was desired that the growth of the organisms from small inocula should not be inhibited by the dye and that the liquid of condensation in the culture medium should not be colored.

The following dyes either partially or completely inhibited the growth of the tubercle organism: 0.05% neutral red, 0.2% brilliant cresyl blue, 0.01% Janus green, 0.2% toluidine blue, 0.2% methylene blue, 0.2% night blue, 0.2% aniline blue, 0.2% magenta or rosaniline, and 0.2% aniline green. For best contrast the authors suggest the use of a dark colored medium, such as blue or black. For this purpose the following dyes may be used: 1.0% trypan blue, 1.2% Pontamine P. G. (DuPont), 1.0% direct fast black and 1.0% direct fast black C and R double (Ciba), 1.0% carbide fast black (Ciba), 1.0% Pontocyl blue black (DuPont) and 0.2% fast acid blue (Coleman and Bell).—*L. Farber.*

DAWSON, VIRGINIA T., and DAWSON, ROY C. Further observations on the use of rose bengal for the enumeration of soil fungi. *Soil Sci. Soc. Amer. Proc.*, **11**, 268-9. 1947.

The effect of rose bengal, when added to glucose-nitrate-soil-extract agar in a final concentration of 1:15,000, on the development of a number of fungi was studied. Some of the organisms were recent isolates from soils and decomposing plant residues while others had been carried on artificial media for a long time. One or more species from the following genera were used: *Alternaria*, *Aspergillus*, *Cephalosporium*, *Chaetomium*, *Cladosporium*, *Coprinus*, *Fusarium*, *Helminthosporium*, *Hormodendrum*, *Penicillium*, *Rhizopus*, and *Trichoderma*. The only evidence of fungistatic activity of the dye in the concentration used was a reduction in colony size. None of the fungal counts were lowered appreciably. The smaller colonies increased the accuracy of the plate count by reducing the inhibitory effect due to crowding and by promoting the formation of discrete colonies. Spreading was considerable in *Rhizopus* and *Trichoderma*.—*R. C. Dawson.* (Courtesy *Biological Abstracts*).

DUFRENOY, J., and PRATT, R. Cytochemical mechanisms of Penicillin action. III. Effect on reaction to the Gram stain in *Staphylococcus aureus*. *J. Bact.*, **54**, 283-9. 1947.

Cells of *S. aureus* under the influence of bacteriostatic concentrations of penicillin gradually lose their positive reaction to the Gram stain.—*Virgene Kavanagh.*

FREDERICQ, PIERRE. Eosin methyl-green-sulfite agar: A modification of Levine's E.M.B. agar. *J. Bact.*, 54, 662-3. 1947.

The toxicity of methylene blue, in Levine's eosin-methylene-blue agar, prevents the growth of some members of the genera *Salmonella* and *Shigella* although this medium is perhaps the best one for isolation and characterization of the coli-aerogenes group. Since the reduced forms of aniline dyes are less toxic than their colored forms, methyl green reduced with sodium sulfite has been substituted for methylene blue. The agar should be prepared as follows: To 1 liter distilled water, add 10 g. proteose peptone, 25 g. lactose (Difco), and 15 ml. 1% methyl green; decolorize until only faint color remains with 10% sodium sulfite adding a drop at a time (will require 1.5-2.0 ml.); add 7.5 ml. 2% eosin Y and 15 g. agar; boil to dissolve completely; sterilize at 15 lb. for 15 min. (No information on dye batches was supplied.)—*Virgene Kavanagh.*

HUBER, WILLIAM M., and CAPLIN, SAMUEL M. Simple plastic mount for preservation of fungi and small arthropods. *Arch. Dermat. & Syph.*, 56, 763-5. 1947.

Stock solution of polyvinyl alcohol ("PVA"): 15 g. of PVA (grade Rh-349) powder is added slowly to 100 ml. of cold water in a beaker. Stir and heat in a water bath at about 80°C. Continue heating until the solution attains the viscosity of thick molasses. Should undissolved clumps or particles remain, filter through two layers of cheese cloth. At this stage the solution may appear milky due to the inclusion of air bubbles. It will later clear on further heating or on standing. This stock solution may be stored. The mounting medium is prepared by combining PVA stock solution 56%, phenol 22% and lactic acid 22% by volume. In mixing, the lactic acid must be added first to the PVA stock solution before phenol is added, otherwise the PVA will change into a soft white sticky mass. In the mounting of cutaneous fungi, cotton blue dye (aniline blue, W.S.) is added to the lactophenol plastic in the proportion of 0.05%. The dye provides a satisfactory degree of contrast in showing up fungus structures. The material to be mounted is placed on a clean dry grease-free slide and a small quantity of the plastic is added, either the colorless medium or a medium to which the cotton blue dye has been added, and a cover slip is pressed into place. It is desirable to have some of the mounting medium flow out around the edges of the cover slip as this aids in the sealing process. Within 24 to 48 hr. excellent clearing of the specimen will have taken place and the specimen will have been sealed in a semihard clear mount. A considerably longer period is required before the plastic becomes completely hardened and permanent in nature. In mounting material removed from fungus cultures, a small amount of agar containing the fungus is removed and placed on a slide and the PVA lactophenol is allowed to flow over and about it. If the cotton blue medium is used, it is allowed to act for 30-60 sec. before the cover slip is placed, in order to allow the fungus structures to absorb the stain. The dehydrating and clearing action of the medium takes care of the agar and its water content, and a permanent mount is provided for the fungus as it grows *in situ*. In slide cultures it is often possible to mount the fungus intact as it grows in culture by cutting out a thin section perpendicular to the surface. A convenient "knife" for this purpose is provided by sealing a small piece of triangular-shaped platinum foil into the end of a glass rod which has been previously softened in a Bunsen flame. A useful tool for transferring fungus material may be made from an inoculating needle inserted into a bacteriological loop holder. The tip of the needle is heated in the flame and bent about 4 mm. to a right angle. Semitransparent mounts of skin (thin scales) to demonstrate fungi may be prepared by transferring these scrapings directly from 70% alcohol to the lactophenol plastic. In mounts of small arthropods, such as *Sarcoptes scabiei* or lice and their ova, the PVA lactophenol clears the object producing a transparency clearly revealing the anatomical structure. For small arthropods with considerable body thickness, hollow ground slides and round cover slips are best. In all these preparations care should be taken to add a sufficient amount of medium initially to allow for loss of substance due to evaporation of the water through drying during the first few days.—*J. A. Kennedy.*

KNAYSI, G., and BAKER, R. F. Demonstration, with the electron microscope, of a nucleus in *Bacillus mycoides* grown in a nitrogen-free medium. *J. Bact.*, 53, 589-59. 1947.

By growing *Bacillus mycoides* on a nitrogen-free medium, consisting of glucose and sodium acetate, until the cells were freed of volutin (ribonucleic acid), bacteria so translucent that their structure could be observed clearly with the ordinary electron microscope were obtained. Bodies with distinctly nuclear properties were observed in both vegetative cells and endospores during their formation. The uniform staining and the uniform opacity to electrons of bacterial cells depend upon the presence of ribonucleic acid.—*Virgene Kavanagh*.

KNAYSI, G., BAKER, R. F., and HILLIER, J. A study, with the high voltage electron microscope, of the endospore and life cycle of *Bacillus mycoides*. *J. Bact.*, 53, 525-37. 1947.

With the ordinary electron microscope, *Bacillus mycoides* cells and spores are opaque, but with high voltages considerable structural detail is visible. To prepare a sufficiently dilute suspension of cells on the collodion film, the following method was employed: Seed a hard agar plate with endospores; press a cover slip lightly against the culture after it has been incubated a suitable time; remove the cover slip carefully; invert it; add a drop of distilled water to the material clinging to the slip and let stand a moment. If the culture is young, place a small loop of this suspension on the collodion film; if the culture has grown for several hours, place a loop of the suspension in a new drop of water on a cover slip and use this dilution on the collodion.—*V. Kavanagh*.

OLLETT, W. S. A method for staining both gram-positive and gram-negative bacteria in sections. *J. Path. & Bact.*, 59, 357-8. 1947.

The following modified Twort method gave excellent contrast between gram-positive and gram-negative bacteria and tissues: Fix material in 5% formal-saline. Pass through alcohols and embed in paraffin. Cut sections 3 μ thick. Dissolve off the paraffin and bring down to distilled water.

Stain in aniline crystal violet 3-5 min. Treat with Gram's iodine, 3 min.; pour off iodine, wash quickly in distilled water and blot dry. Destain with 2% acetic acid in absolute alcohol until no more color comes away. The section should be a dirty straw color. Wash quickly in distilled water. Counterstain in Twort's neutral-red-fast-green stain, diluted 1 part to 3 parts distilled water for 5 min. Wash quickly in distilled water. Destain with 2% acetic-acid-alcohol until no more red stain comes away. Clear in xylene and mount. The nuclei are red; cytoplasm, light green; erythrocytes, green; gram-positive bacteria, dark blue; gram-negative bacteria, pink.—*S. H. Hutner*.

PENSO, G. A new method of staining tubercle bacilli. *Ist. Sanita Publica. Rend.*, 3, 375. 1940.

A new method for staining tubercle bacilli is described: (1) 3-5 min. staining in malachite green (1 g. in 12 ml. 95% alcohol + 100 ml. of water + 5 ml. of phenol); (2) washing; (3) decolorization with a 5% Na₂SO₃ solution (not to be used if older than 48 hr.); (4) washing in flowing water for 15 min.; (5) overstaining through a hydro-alcoholic fuchsin solution; (6) washing and drying. Tubercle bacilli are stained green; all the other germs and elements are reddish.—*G. Gramiccia*. (Courtesy *Biological Abstracts*).

PRATT, R., and DUFRENOY, J. Cytochemical mechanisms of penicillin action. II. Changes in reaction of *Staphylococcus aureus* to vital dyes. *J. Bact.*, 54, 127-33. 1947.

Neutral red, methylene blue, and methyl green normally accumulate in the vacuolar system of bacteria, but in the swollen cells of *Staphylococcus aureus* treated with bacteriostatic dosages of penicillin, these dyes tend to be distributed peripherally. When cup-plates of *S. aureus* are flooded with a saturated aqueous solution of Nile Blue for 1 min., most of the plate, where the bacterial colonies are normal, is blue; but the inhibition zones are purple.—*V. Kavanagh*.

PRATT, R., and DUFRENOY, J. IV. Comparative responses of Gram-positive and Gram-negative bacteria to Penicillin. *J. Bact.*, 54, 719-80. 1947.

The color reactions of the inhibited colonies of the two types of bacteria to a wide range of dyes were almost identical.—*Virgine Kavanagh*.

RUIZ MERINO, J. A method of staining capsules. *Rev. Sanidad e Hig. Publ.*, 20, 1112. 1946.

This easy method requires growing the bacteria under conditions which afford the best development of their capsules. The reagents used are: Solution A: methyl violet, 1%; solution B: distilled water, 10 ml.; horse normal serum, 3 ml. One drop of solution A is mixed with the material to be stained, using a platinum needle, and left 5 min. in a moist chamber to avoid evaporation; then 1 drop of solution B is added, mixing it for 3 min., and a smear like those of hematology is made, using a glass cover slip.—*I. Fernandez*. (Courtesy *Biological Abstracts*).

TOMPKINS, V. N., and MILLER, J. K. Staining intestinal protozoa with iron-hematoxylin-phosphotungstic acid. *Amer. J. Clin. Path.*, 17, 755. 1947.

The use of phosphotungstic acid for differentiating hematoxylin-stained preparations is recommended. The procedure is as follows: Fix 5-10 min. in Schaudinn's fluid in saline solution (a 2:1 mixture of saturated solution of HgCl_2 in physiological saline and 2-5% glacial acetic acid in absolute alcohol, acidified just before use); remove residual mercury by treating 1-3 min. with 70% alcohol containing iodine until of a "port-wine" color; treat twice for $\frac{1}{2}$ min. each with 70% alcohol to remove residual iodine; mordant 3-5 min. in 4% aqueous ferric ammonium sulfate; wash 1 min. in running tap water; stain 1 min. in week-old 0.5% aqueous hematoxylin; remove excess iron by dipping in water; differentiate 2 min. or more in 2% aqueous phosphotungstic acid; blue the hematoxylin by treating 1 min. in tap water or dilute alkali; dehydrate 1 min. each in 95% and absolute alcohol; clear 1 min. in xylene; mount in dammar.—*L. Farber*.

WHITE, P. BRUCE. A method for combined positive and negative staining of bacteria. *J. Path. & Bact.*, 59, 334-5. 1947.

Information as to the encapsulation, sporulation, and viability of bacteria is conveniently obtained by the following technic: Remove grease from a slide by flaming. Place on the slide a drop of an almost saturated solution of Congo red plus 10% of blood serum. Mix in a particle of the agar-grown culture to be examined. Spread the mixture on a film, dry with gentle warmth, and fix thoroughly in a flame. Flood the film when cool with 5% aqueous HCl . Drain, blot, and drive off excess acid with gentle warmth.

Stain for 15-20 sec. with 1% aqueous methylene blue, which may be acidulated with 1 small drop of glacial acetic acid to 20 ml. of stain. Drain off stain (do not wash), blot gently but thoroughly.

The blue-stained bacterial bodies stand out against a background of orange or gold. Capsules appear as haloes, sometimes tinted violet. Interstitial material responsible for pellicle formation is often violet. Spores are very conspicuous because of their refractility. Bacteria alive at the time of preparation of the film are blue; those dead at the time stain brown or purple because of their permeability to the Congo red.—*S. H. Hutner*.

HISTOCHEMISTRY

SEMMENS, C. S. Theory and technique of cyto-microchemistry. *The Microscope*, 6, 197-200. 1947.

This brief article is really but an introduction to the subject indicated by the title. The only phase of the subject treated is that of fixation of tissue and its probable effect on cell chemistry, especially on the chemistry of chromatin. It is pointed out that when a piece of living material is put into a fixing solution the constituents at once lose the normal properties of living matter. It is reasonable to suppose that an ordinary fixing agent may cause molecular modifications similar to those that result from the action of "denaturing" agents on protein extracts in large scale biochemical investigations. The author indicates that the protein of fixed tissue may well be "denatured" protein; but insists that the validity of this assumption still remain to be tested experimentally.—*H. J. Conn*.

STAIN TECHNOLOGY

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RAPID STAINING OF HEINZ BODIES IN SMEARS

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Heinz (1890) was one of the first investigators to describe the occurrence of colorless refractile globules of varying sizes in the erythrocytes of animals following poisoning by certain chemicals, such as phenylhydrazine, its derivatives and anilin. While these globules were often seen without staining, Heinz (1890, 1901) rendered them more easily visible by the addition to the fresh blood preparation

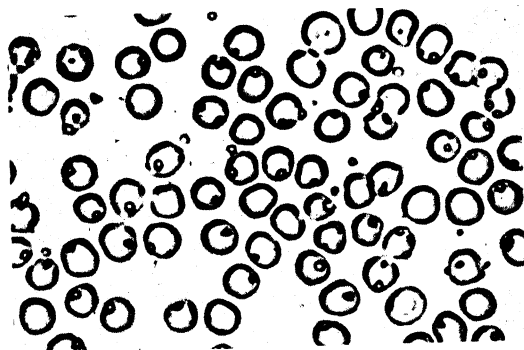


FIG. 1.—Heinz bodies in erythrocytes of mouse following oral administration of sulfanilamide. Smear stained and fixed with alcoholic methyl violet solution. $\times 1100$

of a saturated solution of methyl violet in 0.6% sodium chloride. Later (1904) this was modified to 0.03% solution of the dye in 0.75% salt solution.

Although this supravital method has a number of advantages (Webster, et al., 1948), in that each cell can be carefully examined for Heinz bodies and thus an accurate count can be made, the erythrocytes of some species are rapidly altered when subjected to this treatment.

Heinz body preparations of fixed and stained smears were preferred by Ehrlich (1898) who used his tri-acid stain. Naegeli (1931) advocated the use of 0.5% Nile blue sulfate solution in preference to the usual Giemsa stain which gave uncertain results. However, none of these substances appears to give satisfactory staining.

We have found that the aqueous solutions employed by Heinz will stain the Heinz bodies in unfixed smears but most of the erythrocytes are hemolyzed. Previous fixation with either methyl or ethyl alcohol results in removal of the majority of Heinz bodies from the cells. However, by use of a 0.2% solution of methyl violet (anilin violet, Dahlia B, C. I. No. 680; total dye content 88%) in 95% ethyl alcohol the smears can be fixed and stained simultaneously. In place of this dye, crystal violet (Gentian violet, C. I. No. 681, Certification No. NC 29, total dye content 95%) has been used with equal success.

In use, a freshly prepared, air-dried, moderately thick, blood smear is covered with the methyl violet solution and allowed to stain for one-half minute, following which the slide is washed with running water to remove the surplus dye. The Heinz bodies are visible as blue granules, the depth of color depending somewhat on their size and the thickness of the smear. Since these bodies can be extruded from the cells through pressure, the mechanical trauma involved in preparing the slides may release them. Consequently many extra-erythrocytic bodies are usually found in the thinner portions of the smear.

Fig. 1 is an enlargement of a photomicrograph of a typical Heinz body preparation. This shows the bodies in the erythrocytes of a mouse which had been given 0.3% sulfanilamide in its drinking water.

We wish to thank Mr. E. C. Thompson of this Laboratory for assistance in this work.

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HISTOLOGICAL AND HISTOCHEMICAL USES OF PERIODIC ACID¹

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ABSTRACT.—Periodic acid acts upon the 1,2 glycol linkage ($-\text{CHOH}-\text{CHOH}-$) of carbohydrates in tissue sections to produce aldehyde ($\text{RCHO}+\text{RCHO}$) which can be colored with Schiff's reagent. The method can be used on frozen or paraffin sections and is useful as a reaction for carbohydrates of tissues: glycogen (in paraffin section only), mucin, basement membrane, reticulin, the colloid of the pituitary stalk and thyroid, some of the acidophile cells of the human anterior hypophysis, the granular cells of the renal arteriole, etc.

In abnormal tissues, it colors many of the "hyaline" materials—amyloid infiltrations, arteriosclerotic hyaline, colloid droplets, mitotic figures, etc.

The histochemical uses of the periodic-acid-Schiff's reagent (PAS) need careful control because of the possibility of attachment of iodate or periodate to tissue constituents, producing a recoloration of the Schiff's reagent. Whenever possible the positive reacting material should be further identified by other methods since Lison showed other substances besides aldehydes can recolorize Schiff's reagent.

This paper reviews the histological and histochemical uses of periodic acid. It presents for the first time the technic for frozen sections.

Carbohydrates and carbohydrate compounds can be demonstrated in microscopic sections of tissue by the action of a solution of periodic acid (Hotchkiss, 1945²; McManus, 1946, 1947, to be published; Lillie, 1947 a & b.) The aldehyde formed from carbohydrates by periodic acid is colored with Schiff's reagent as in Feulgen's test, (Feulgen, 1924.) Malaprade (1934) found that periodic acid acted upon 1,2 glycols ($-\text{CHOH}-\text{CHOH}-$) to form aldehydes, and used the method in quantitative analyses of alcohols. Nicolet and Shinn (1939) demonstrated that aldehyde is formed by periodic acid in 1,2 glycols even if one hydroxyl group be substituted by an amino group

¹The investigation at the Medical College of Alabama was aided by a grant from the Life Insurance Medical Research Fund.

²Work done in U. S. Naval Reserve, reported in 1945, but not published. See Hotchkiss (1948).

as in the amino acids, serine, threonine and hydroxylysine. The necessary linkage does not appear in the combined amino acids of tissue proteins while the free amino acids are water soluble with the possible exception of hydroxylysine.

Hotchkiss appears to have been the first to use periodic acid on tissues for carbohydrates. He has made the most complete analysis of the chemistry involved as will appear later. (Hotchkiss, 1948). The first publication describing the use of periodic acid (McManus, 1946) dealt with the demonstration of mucin. It was mentioned that the reaction colored also "certain cells in the pituitary, colloid of the pituitary stalk and thyroid, granules in some nerve cells in the medulla of the rat and the human intestine and the basement membranes of the tubular epithelium and of the glomerulus". More recently, Lillie has used periodate and nitric acid (periodic acid) as a basis of a similar reaction for reticulum and basement membranes (1947a) and for glycogen (1947b).

The major advantage of periodic acid for use in histochemistry is the known specificity of the reaction upon the 1,2 glycol linkage. The use of chromic acid in Bauer's (1933) "test" for glycogen was developed in an empirical fashion³ and the exact reaction involved is unknown, as Hotchkiss points out. Similarly the use of potassium permanganate by Casella (1942) and by Lillie (1947b) and of sodium persulfate by Bignardi (1946) to produce substances colorable with Schiff's reagent appear to produce interesting results, the full significance of which is not now apparent.

The demonstration of carbohydrates in tissues depends primarily upon the carbohydrates normally present and their fate in fixation and dehydration. There has been a reversal of feeling on the effect of water on tissue carbohydrates. The studies of Lison (1936) have shown that aqueous solutions need not be avoided in the preservation of glycogen, for example, into sections. Picric alcohol and various picric acid combinations appear to be the best fixatives for glycogen. Lillie (1947b) has come to similar conclusions. For human tissues, ordinary Zenker-formol or Helly's fixative as described in routine textbooks appears quite satisfactory. The usual dehydration routine of alcohol and toluene or xylene can be followed and one may even dehydrate the tissues in the Autotechnicon. For frozen sections the formol-saline technic of fixation has been used and has been found quite satisfactory. A fat stain may be superimposed upon a periodic

³Many non-glycogen substances, mucin, thyroid colloid, etc., can be shown with Schiff's reagent after the reaction of chromic acid. References: Wallraf and Bechert (1939), Bignardi (1940), Dempsey and Wislocki. 1947).

acid preparation in frozen section using the usual technics of Sudan IV or Sudan black.

Obviously the type and amount of tissue carbohydrates demonstrated by the periodic-acid-Schiff's-reagent (PAS) technic will depend to some extent upon the species of animal and upon the type of handling. Descriptions and routines which we will present here will be taken to refer to paraffin sections of human Zenker-formol fixed tissues. In the frozen section technic formol-saline fixation has been used.

PERIODIC-ACID-SCHIFF'S REAGENT (PAS) HISTOLOGICAL TECHNIC

A. ROUTINE FOR PARAFFIN SECTIONS (McMANUS, TO BE PUBLISHED)

1. Paraffin sections to water.
2. Wash in running tap water 5 minutes if iodine and hypo were used.
3. 0.5% periodic acid in water 5 minutes.
4. Rinse in distilled water.
5. Schiff's reagent, 15 minutes.
6. Rinse in three changes of sulfurous acid, each 2 minutes.
7. Wash in running water 3 to 5 minutes.
8. Stain in Harris hematoxylin 20 to 30 seconds.
9. Wash in running water 5 minutes.
10. Dehydrate in two changes of 95% alcohol.
11. Two changes of absolute alcohol.
12. Clear in xylene and mount in balsam.

(Note: Steps 8 and 9 are optional.)

Preparation of Schiff's Reagent

1. Weigh out basic fuchsin—one gram.
2. Weigh out anhydrous sodium bisulfite—one gram.
3. Boil 200 ml. distilled water.
4. Add fuchsin and stir.
5. Cool to 50°C.
6. Filter.
7. Add 20 ml. N HCl. (98.3 ml. of HCl, S.G. 1.16, made to 1000 ml. with distilled water.)
8. Cool to 25°C.
9. Add sodium bisulfite.
(Keep in dark. The fluid takes about two days to become orange or straw colored; then it is ready for use.)

Sulfurous Acid Rinse

1. 10% sodium metabisulfite—6 ml.
2. Normal HCl—5 ml.
3. Distilled water—100 ml.

B. Routine for Frozen Sections

Frozen sections to 0.5% aqueous periodic acid—5 minutes.
Wash in several changes of distilled water.

Schiff's reagent—10 minutes.

Rinse in three changes of sulfurous acid, each 2 minutes.

Tap water.

Counterstain, if desired, Harris' hematoxylin 20–30 ml.

Wash well in water.

Mount in glycerin, or glycerin jelly, or dehydrate and mount in balsam.

Notes: These solutions keep their potency fairly well. The periodic acid solution is kept in a covered Coplin jar in a cabinet up to a month with moderate use, without apparent loss of strength. The Schiff's reagent deteriorates more rapidly. This is manifested by a return of the red color to the solution and a weakening of the coloration of the sections. Lillie (1947b) suggests keeping the Schiff's reagent in the icebox to preserve its potency.

It has been our custom to discard the first sulfurous acid rinse when a new batch of slides is being carried through the procedure. The second rinse is moved up to the first, the third to the second place and a new third rinse is made up. That means that with each set of slides the last two minutes of the rinsing is spent in new sulfurous acid, containing not a trace of Schiff's reagent, while the first two rinses are still colorless and potent. Hematoxylin-eosin sections have been used for PAS coloration up to 25 years after the original preparation. The cover slips are soaked off in xylene at 37°C. and the sections are brought down to water. A minute or so is sufficient to bleach the section in 1% HCl in 70% alcohol and the sections are washed in running tap water for 5 to 10 minutes. The ordinary routine for paraffin sections (A above) is followed. Glycogen, mucin, etc. can be seen in the old sections.

HISTOLOGICAL RESULTS

The *frozen section* technic does not appear to show glycogen in sections where it can be shown in corresponding paraffin sections. The other material to be described—mucin, reticulin and basement membranes—color equally well in frozen section. The arteriolar granular cells are better shown in frozen sections in two cases of malignant hypertension. The outer part of the myelin sheath (? neurokeratin) does not appear PAS positive in paraffin sections but is seen in frozen section. The *paraffin sections* after PAS treatment show a coloration of known carbohydrates of tissues, that is, glycogen, mucin and hyaluronic acid, if they have been fixed adequately and within 12 hours of death. (Hyaluronic acid is somewhat better preserved after overnight fixation in 4% subacetate of lead.) There are shown, as well, reticulin, fibrin of thrombi, colloid droplets, the hyaline of arteriosclerosis, hyaline deposits in glomeruli as in intercapillary glomerulosclerosis, the granular cells in the renal arterioles where preserved, most basement membranes, the colloid of the

pituitary stalk and thyroid, some of the acidophil cells in the anterior lobe of the hypophysis, amyloid infiltrations, etc.

Combination colorings involving the PAS routine are possible. The staining of fat after the periodic coloring of frozen sections and the nuclear counterstaining of frozen and paraffin sections have been mentioned. Other stains for paraffin sections follow the same principle, coloring after the PAS, with the exception of the combined Feulgen-PAS routine.

The Feulgen hydrolysis with 2 *N* HCl at 60°C. for 5 to 10 minutes (followed by rinsing in distilled water) is introduced before the periodic acid oxidization, for example: Paraffin sections to water; wash in running tap water 5 minutes if iodine and hypo were used; 2*N* HCl at 60°C. for 4 to 10 minutes; wash tap water; 0.5% periodic acid in distilled water 5 minutes; Schiff's reagent, 30 minutes. Follow on as A above (p 101) omitting steps 8 & 9. The usual routine is followed but the Schiff's reagent time is increased to 30 minutes. The result is a histochemical preparation with most of the features of the routine histological section.

Lillie (1947b) has mentioned counterstaining with dilute picric alcohol after the nuclear stain. We ordinarily use no cytoplasmic stain. The usual Prussian blue stain for iron can be done after the PAS routine. The positive staining is usually blue-red to violet, some carbohydrate being incorporated into the pigment granules. An elastic tissue stain with acid orcein can be done on PAS colored paraffin sections, followed by Harris' nuclear hematoxylin.

THE HISTOCHEMICAL USES OF PERIODIC ACID

The explanation of the chemical basis for the use of periodic acid in histochemistry has been done most thoroughly by Hotchkiss (1948). Since the 1,2 glycol linkage ($\text{R}-\text{CHOH}-\text{CHOH}-\text{R}$) is split by periodic acid to form aldehyde ($\text{R}-\text{CHO}+\text{R}-\text{CHO}$) there must be the following requirements to give a positive coloration: (1) preservable material with the proper linkage must be present, (2) the RCHO groups must not be diffusable, that is the aldehyde must be attached to a non-diffusable substance and (3) concentration of the final aldehyde-fuchsin compound must reach a certain level of concentration in section.

Hotchkiss (1948) introduces a reducing rinse "to remove entrapped or combined periodate or iodate" since either salt will re-colorize Schiff's reagent. As a substitute we have used control sections left in the corresponding solutions—0.5% aqueous sodium periodate or 0.5% aqueous potassium iodate for 5 minutes—without any coloring subsequently in Schiff's reagent.

It would appear further that Hotchkiss' reducing rinse actually decreases the amount of aldehyde which is available for coloring the Schiff's reagent or otherwise interferes with the brilliancy of the stain. If a Feulgen test is carried out and Hotchkiss' reducing rinse is interposed between the hydrolysis and the Schiff's reagent, the subsequent coloration of the desoxyribose nucleic acid is reduced considerably and it will almost disappear if the reducing rinse is prolonged for 15 minutes.

In summary, the use of the reducing rinse appears to decrease the sensitivity of the PAS reagent test. For example, glycogen is shown in good relief by the Hotchkiss method because the other tissue carbohydrates—basement membranes and reticulin, etc.—show so poorly. The glycogen apparently has so many reactive points that a reduction in the total number of the aldehyde groups does not much reduce the subsequent coloration with Schiff's reagent. Other tissue carbohydrates, however, produce less aldehyde after periodic acid and the action of the reducing rinse appears frequently to be the near extinction of the coloration which appears in the structures if Schiff's reagent follows directly upon the periodic acid.

As Hotchkiss points out, his unpublished method has been utilized in histochemistry in two different sets of work. The identification of PAS positive material in pituitary cells as FSH (Catchpole, 1947) and the PAS material in the thyroid colloid as thyreoglobulin (Gersh, 1947) have made use of freezing-drying technics and isoelectric point determinations. Similar procedures might aid in identification of other substances which, like the thyroid colloid and the pituitary cells, have been described with mucin as PAS positive (McManus, 1946).

DISCUSSION

The further identification of the carbohydrates in tissues which have been shown by the periodic acid routine is the present crux of the situation. Glycogen can be identified by its removal with saliva or with diastase as Lillie (1946) has pointed out. The use of other enzymes or carbohydrates such as hyaluronidase and glycosidase or emulsin may be expected to offer some promise. In the hands of Dempsey and Wislocki (1947) hyaluronidase has not proved successful in removing carbohydrates shown with one or another oxidizing test but the metachromatic staining of the hyaluronic acid is lost in the procedure.

Some bacteriological investigations with the aqueous PAS technic, as we originally described it, have been interesting. It will be recalled that Hotchkiss (1948) and Lillie (1947) have not been able to

color certain bacteria (pneumococcus and streptococcus) with periodic acid followed by Schiff's reagent. Dr. C. H. Winkler in the Department of Bacteriology (personal communication) has been able to color the metachromatic granules of *Corynebacterium diphtheriae* and diphtheroids quite strongly while bar and granule formations appear in some of the *Salmonella enteritidis* organisms.⁴

Pectin and various corresponding animal tissues, chitin for example, might be expected to color with the periodic acid routine. Limited studies of fungi show strong coloration and further studies have been reported in moderately extended form by Lillie (1947). None of these materials in the bacteria can be taken to be carbohydrates until they have been tested with Hotchkiss' reducing rinse or, as an alternative, soaked in solutions of potassium iodate and sodium periodate for a time corresponding to that in which the periodic acid acts on the bacteria.

An interesting finding with the PAS routine in paraffin sections of Zenker-formol fixed tissue from the human duodenum is the coloring of the free surface or the cuticle of the intestinal epithelial cells. This happens to be the site of activity of alkaline phosphatase and one sees also some coloring of the brush border of the proximal convoluted tubular epithelium in the kidney, also a site of alkaline phosphatase. There is a localization of the positive reaction in the PAS routine in human mitotic figures, particularly in tumors. We have not studied mitotic figures in normal material in sufficient number to be sure that there is not an occasional strong coloring of the mitosis of the normal cell. This again may be a site of phosphatase activity. We have not studied mitotic figures in any animal tissues or tumors.

The study of periodic acid preparations of frozen sections of human kidney shows many features not obvious in paraffin sections. The structure of the glomerulus is particularly well shown and the curving of each set of capillary loops around a central core in the glomerulus can be made out well, particularly in thicker sections. The appearances in diabetic glomeruli are interesting. The central core even in the absence of frank intercapillary glomerulosclerosis still shows a "hyaline" rather than the "fibrillar" structure which one is accustomed to see in the non-sclerotic diabetic glomeruli in paraffin sections.

The frozen section of the human adrenal—the only species we have studied—shows an accumulation of PAS positive material in

⁴With PAS, a paraffin section of kidney infected with *Clostridium welchi* has loss of the basement membranes where the organisms are present. The clostridia show coloring of their capsules with PAS. This is the only organism which we have colored in sections with the PAS technic.

globular form up to 1–2m μ in diameter in the reticular zone or in the outer portions of the medulla.

As Lillie has already pointed out, the use of PAS for reticulum of the spleen in paraffin sections produces probably the best results of any method for studying splenic reticulum. It is quite as selective in frozen section. We have used a variety of normal and abnormal spleens in a limited study of the matter and the changes which are the result of sickle cell anemia, chronic passive congestion, and tuberculosis for example, are well shown.

In lymph nodes the reticulum is colored with the periodic acid routine on paraffin section. Tumor metastases in lymph nodes are well demarcated and one occasionally finds a suggestion of origin of tumor metastasis from the coloration of mucin.

The comparative carbohydrate content of giant cells in various granulomatous processes would be worth studying.

It seems reasonable at the present time to differentiate fairly sharply between the histological and the histochemical uses of periodic acid. The greatest histological use of the PAS routine will be that of the simplest technic which I believe is the first described (McManus, 1946) and herein repeated in greater detail. It is a method for connective tissues which can be used on paraffin sections or on frozen sections, and in addition shows many or all of the tissue carbohydrates—glycogen sometimes, mucin always, basement membranes, reticulin etc.—after almost any fixing method. It has the advantage of needing no differentiation and can be done well by the veriest tyro among technicians on first attempt.

The histochemical use of periodic acid should be restricted to the study of carbohydrates in sections where the carbohydrate nature of the material can be corroborated by some further procedure, e.g. glycogen is removed by diastase, since Lison showed acetone and unsaturated acids can recolorize Schiff's reagent. The routine should be carefully controlled by the use of Hotchkiss' reducing rinse or by soaking corresponding sections in 0.5% potassium iodate and 0.5% sodium periodate at the time that the test section is in periodic acid. The three sections are then carried through the Schiff's reagent and subsequent treatment together.

The use of periodic acid in histology and histochemistry is not yet two years old. The obvious use is for glycogen (Marchese, 1947). The most interesting results are those in which the carbohydrate content has been presumed—thyroid colloid, FSH, amyloid infiltration, basement membranes and inter-cellular substance—or in which new data are presented—mitotic figures.

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THE RAPID PREPARATION OF FROZEN TISSUE SECTIONS

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ABSTRACT.—The essential feature of this procedure involves the rapid freezing of the tissue following excision and keeping it frozen until the desired chemical or fixative has been applied. For freezing, either carbon dioxide or liquid air is used, as desired. The microtome knife is thoroughly cooled by taping blocks of dry ice to its surface. The cut sections, still frozen, are manipulated by a camel's hair brush so that they lie flat upon the knife. They are then transferred to a slide by a special section lifter. This has the form of a double-bottomed scoop packed with dry ice. Thus the section remains frozen while it is transferred to a clean microscope slide held at an angle above a Coplin jar of the desired reagent. The sections must be immersed just prior to melting. They curl and do not adhere to the slide if still rigidly frozen, and are distorted if immersed after melting.

With this technic sections showing a minimum of cellular distortion may be obtained. Consequently, it facilitates the use of many cytological technics, chemical tests, and enzymatic studies, such as the Gomori technics, on a variety of tissues.

The revival of Altmann's (1890) freezing-drying technic, Gersh (1932), Bensley and Gersh (1933), brought into prominence once more a very valuable method for handling tissues for histological and cytological investigations. The method as finally elaborated and improved by them consists of the very rapid freezing of the material followed by dehydration for some twelve hours or more *in vacuo* and lastly the direct embedding of the tissue in paraffin after a short infiltration with melted paraffin. While the method has obvious advantages, it is almost impossible to use in laboratories that are not equipped for carrying out such intricate procedures. This situation confronted the authors in the course of certain studies on nervous tissues. Furthermore, we wished to avoid not only the solvent action of water which is encountered when the usual procedures are followed in floating out frozen sections before mounting them on slides, but also any other chemical changes which might be induced by the use of alcohol, hot paraffin, etc.

Our final solution of these difficulties was to keep the material frozen from the moment it was placed on the block-holder of the

microtome until the sections, still frozen, were affixed to the slide and treated with the desired reagents, Adamstone and Taylor (1947). Because the method has a number of advantages, it is described in detail for the benefit of other workers who may wish to make use of it. Attempts to develop such a method have been made by a number of investigators, and some of the early trials are described by Becher in the Leitz Manual on the use of the microtome (3rd edition).

Apart from the fact that when the tissue has once been frozen it must never be allowed to melt and be refrozen during the entire procedure, the first prerequisite for the successful adoption of this technic is that the microtome knife be kept exceedingly cold. This was accomplished by placing blocks of dry ice on each side of the spot on the knife where the sections would lie when cut. The dry ice can be kept in place by attaching a fence made from a piece of light tin to the microtome knife or by taping the ice in place with pieces of Scotch tape. This cools the blade so that the sections do not melt when they rest upon the knife. By using a very fine camel's hair brush, the sections may be prevented from curling and may be gently eased up on the knife where they lie frozen and spread out almost flat. The microtome used for this work was a Leitz base sledge type operated by hand. The microtome knife was a very heavy one (10 inches long) adjusted to a position at right angles to the direction of movement of the block of tissue. Carbon dioxide from a pressure tank was used to freeze the tissues. The materials to be sectioned were removed rapidly from the animal in the form of relatively thin slices (2 mm. or less) and frozen immediately. Such thin slices of tissue not only cut better, but thinner sections may be obtained since the temperature can be maintained at the proper level more easily.

The second precaution to be observed for the use of this method is that the sections must be transferred *while still frozen* to a microscope slide. For this purpose a special section lifter was constructed of tin in the form of a double-bottomed scoop (Fig. 1). The space between the bottom of the section lifter and its upper surface on which the sections lie, was enclosed at the sides, thus forming a small compartment that could be filled with chips of dry ice. Using a camel's hair brush, the sections were transferred from the microtome knife to the section lifter and then to a thoroughly clean microscope slide kept at room temperature.

In the third place, it is *essential that melting of the sections be prevented* until chemical tests are started or a fixative is applied to preserve the section for later processing. The application of fixatives or

various solutions must be done at *exactly the right moment*. This is the instant at which the section just begins to melt and flatten on the slide. If the fixative is applied too soon before this occurs and while the section is still rigidly frozen, the tissue shrivels and curls up the moment the fixative comes into contact with it. If applied too late, after the section has completely melted, the fixed cells are ruptured or distorted. The most important single item in producing first class preparations is the rapid immersion of the sections in the desired fixative or other solution at exactly the proper moment just as melting occurs.

The handling of the sectioned material is managed much more efficiently by two people working together. One holds the slide at a slight angle above the solution which is contained in a Coplin jar. The other transfers the frozen section to the slide which is instantly dropped into the liquid by the first worker. This method has the added advantage that the slide comes to rest in a vertical position, and as a result, most of the minute air bubbles which might be trapped are forced out of the material. In some cases it is desirable to apply the fixative or other solution to the section from a pipette while the slide is in a horizontal position. The sections stick firmly to the slide and after fixation may be left for some time in appropriate solutions until it is convenient to work with them. Completed stained sections may be mounted in Clarite or other suitable medium as the methods dictate.

The advantages of the method are numerous—one of the most important being the rapidity with which fixation and other processing may be carried out. For example many standard technics may be completed in a few minutes. Other cytological technics which ordinarily require prolonged treatment over a period of days or weeks, e.g., the Gomori enzyme procedures, may be completed in a matter of hours, or less. Furthermore, it is possible to use certain technics on large sections of tissue although these methods can ordinarily be applied only to very small blocks of tissue when the usual cytological methods are employed. In some cases, considerable experimentation may be needed to work out routine methods. While our experience has been largely confined to work with brain and liver material, many other tissues have been used and it has been found that the method has a considerable number of useful applications including chemical studies, enzyme analyses, and probably also micro-incineration, the use of radio active materials and a variety of other procedures.

Certain difficulties are encountered in using this technic. For ex-

ample, it is impossible to work in a warm room, but a cool basement room, air conditioning or winter temperatures will help to overcome this difficulty. Furthermore, humid weather conditions seem to interfere with the work since moisture condenses on the sections and they do not adhere readily to the slide. In addition, it has been found that certain tissues are inherently very difficult to cut and handle, e.g., muscle which retains contractility for long periods.

This technic may be modified so that the tissue is first fixed, before the frozen sections are cut. The adoption of this method loses the advantage of working with unfixed tissue and perhaps the only advantage to be gained is that when fixed tissue is used it is not necessary to handle the sections with the same painstaking care that is necessary when working with unfixed frozen sections.

DISCUSSION

By comparison of the present method with the modification of Altmann's technic introduced by Bensley and Gersh (1933), it is apparent that by the use of liquid pentane they are able to accomplish much faster freezing than is possible by the use of carbon dioxide. They have thereby sought to avoid the destruction of the finer cytoplasmic structure of the cell caused by the formation of relatively large ice crystals which is said to occur when freezing takes place at the higher temperature of solid carbon dioxide. Experiments on this phase of the subject were carried out by Hardy (1899) and Hoerr (1943), who maintain that lower temperatures produce a more normal condition of the cells. (See Hoerr's paper for further references.) We have also frozen tissues at the temperature of liquid air ($-190^{\circ}\text{C}.$) and have kept them frozen for sectioning by the use of CO_2 ($-78^{\circ}\text{C}.$). Obviously no change in the ice crystals formed at $-190^{\circ}\text{C}.$ can take place as these crystals warm up to the temperature of CO_2 ($-78^{\circ}\text{C}.$) other than the infinitesimal expansion of the crystal occasioned by this change in temperature. Sections prepared in this manner and examined with the best powers of the microscope available to us appear to be identical with those frozen directly with CO_2 . We are of the opinion, therefore, that the difference in the amount of distortion produced by ice crystals formed at the temperature of liquid air and those formed at the temperature of solid CO_2 is undetectable with our best microscopic equipment when sections are prepared by the methods outlined above.

Bensley and Gersh (1933) recognize the possibility of the solvent action of hot paraffin in their technic. The present method not only eliminates entirely the use of heat but also the use of melted paraffin and any changes caused thereby. It must be emphasized once again,

however, that the material must be kept frozen *continuously*, and that fixation or treatment with chemicals must be started without delay. Melting is disastrous. If these precautions are observed, it is evident that the extreme thinness of the sections (down to 4μ) used in this work makes possible exceedingly rapid penetration of fixatives or chemicals used in various tests.

EVALUATION OF METHOD

In order to make a satisfactory evaluation of the worth of this method, sections prepared in this manner must be compared with: a) living cells, b) fresh unfixed frozen sections mounted in saline, and c) with preparations made according to standard technical procedures. In Fig. 2, living cells teased from a thin slice of liver from the hamster are shown stained with Janus green B and mounted unfixed in saline. These cells are rounded up to some extent but in the cytoplasm small fat droplets are visible, each surrounded by a more or less complete rim of material densely stained with Janus green B. Fig. 3 shows a small portion of a frozen section of fresh liver from the same animal prepared by the methods described in this paper. It may be compared with the fresh cells in Fig. 2 and with the fixed stained section of frozen material shown in Fig. 4. In this unfixed section which was also stained with Janus green B, cells of the hepatic cord are seen to contain numerous fat globules again surrounded by material stained with Janus green B. Under the microscope the nuclei are also clearly visible even though they do not show in the photograph. The frozen section (Fig. 4) was placed on the slide, fixed in formalin, stained with Sudan IV to demonstrate the presence of fat, and treated with silver nitrate, and a cytoplasmic counter stain of light green. In this section the fat droplets stain a very conspicuous brilliant red color and each is surrounded by a heavy rim of granules stained black by the silver. The nuclei of the cells are also clearly visible.

The sections illustrated in Fig. 5, 6, 7, and 8 were photographed with a high dry objective in order to show a larger field so that the frozen sections may be compared with a section prepared by the standard paraffin method (Fig. 5). All sections were stained with Harris's hematoxylin and counterstained with orange G. The material shown in Fig. 5 was fixed *in toto* in cadmium formol. The fresh frozen sections, after being placed on the slide, were fixed in cadmium formol (Fig. 6), 95% alcohol (Fig. 7), and F. A. A. (Fig. 8). It is quite evident that the frozen sections fixed on the slide show less shrinkage of the nuclei than occurs in the standard preparation. The cytoplasm of both standard and frozen sections,

in general, appears very similar but actually differs in that the cytoplasm of the latter is perhaps more homogenous and contains many small bodies (seen only with the oil immersion objective), some of which stain prominently with hematoxylin or may be readily impregnated with silver nitrate as shown in Fig. 9.

It is quite obvious from a study of the photographs presented that sections prepared according to the methods described herein are equal if not superior to the sections prepared by the usual standard procedures and probably give a truer and more exact concept of the finer structural details of the cell. In addition, such sections may be so treated as to bring out many cytological details which can only be seen in standard preparations after the application of long, tedious, and often unreliable methods. In fact, the potentialities of the method are becoming more apparent and more interesting as a result of greater experience with it. We have attempted to emphasize the fact that the utmost care must be used in preparing materials according to the methods we have described. If this is

PLATE 1.

FIG. 1.—Section lifter with compartment for dry ice.

FIG. 2.—Liver cells of mouse ($\times 850$) teased apart and stained with Janus green B. Fat droplets appear clear and are surrounded by a more or less complete rim or granules stained with Janus green B. Note that isolated cells tend to round up.

FIG. 3.—Portion of a frozen liver section of mouse ($\times 850$), unfixed, but stained with Janus green B. Fat droplets appear as clear vesicles and are surrounded as in Fig. 2 by a rim or granules stained with Janus green B.

FIG. 4.—Portion of frozen liver section (hamster). Fixed in formalin, treated with silver nitrate and stained with Sudan IV and light green ($\times 850$). Fat droplets of varying size appear dark in close association with silvered bodies. Nuclei can also be seen.

FIG. 5–8.—Sections of mouse liver stained with Harris' hematoxylin and orange G ($\times 400$).

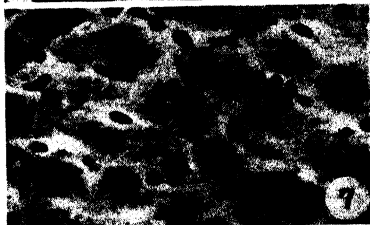
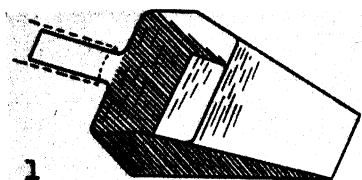
FIG. 5.—Standard paraffin section of material fixed in cadmium formol.

FIG. 6.—Frozen section fixed in cadmium formol.

FIG. 7.—Frozen section fixed in 95% alcohol.

FIG. 8.—Frozen section fixed in formalin, alcohol, and glacial acetic.

FIG. 9.—A single liver cell from hamster ($\times 1700$) after treatment with silver nitrate. Nucleus, silvered bodies and a few black-rimmed fat droplets can be seen.



not done poor sections will be obtained just as occurs when the paraffin method is improperly used.

SUMMARY

A technic is described for making and handling frozen tissue sections whereby the fresh unfixed material is kept continuously frozen until sections are affixed to microscope slides for processing. The steps in this process are:

1. Rapid removal of the tissues from the animal and immediate freezing with liquid air or carbon dioxide.
2. Thorough cooling of the microtome knife with blocks of dry ice.
3. Handling of the sections by means of a scoop-like double-bottomed section lifter filled with dry ice chips.
4. Transfer of the frozen section to a microscope slide.
5. Rapid immersion of the slide in the fixative or other fluid just as melting begins.

The method avoids the use of hot paraffin and the effects of all other unwanted chemical substances. Since the sections are very thin penetration by the fixatives or chemical employed is very rapid. As a result a great variety of chemical methods may be used on the fresh tissues, or, the usual cytological and histological staining technics may be employed.

The sections, when properly prepared, are of very fine quality.

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MANIPULATION OF TISSUES FOR IMPROVED ACETO-CARMINE STAINING

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The importance of the study of the structural-mechanic properties of any particular material in order to improve the technic for aceto-carmine smears and to obtain better preparations of that material has not been, perhaps, sufficiently emphasized in the large number of papers on such cytological technic. The usefulness of such a study will be shown here in two cases met by the writer.

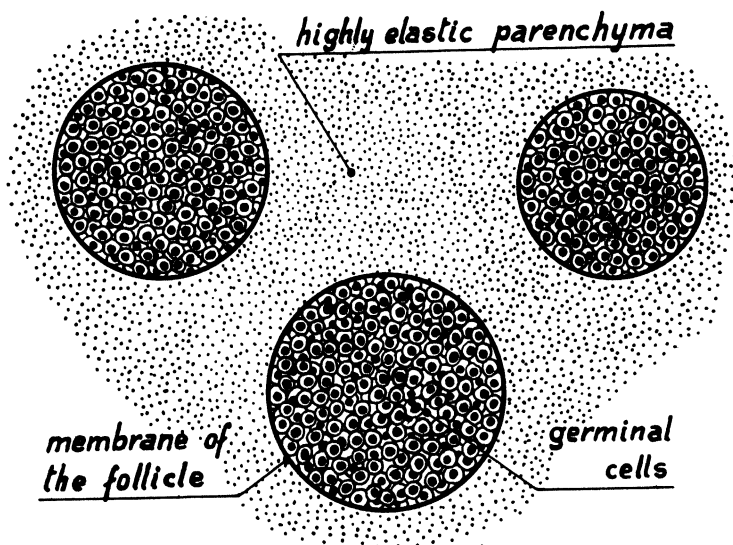


FIG. 1.—*Thysanozoon brocchii*: parenchyma with male germinal cells (a sketch).

The first one concerns the preparation of the male germinal cells of *Thysanozoon brocchii* (Turbellaria, Polyclads). In this worm the testes consist of little follicles (diameter about 150 μ), scattered ventrally in the parenchyma, each one of which is covered by a hard cuticle (Fig. 1). Preparations made by taking small pieces of parenchyma containing some follicles gave very poor results: the follicle membrane was only seldom ruptured and besides the germinal cells spread out with great difficulty in the preparation. This fact was attributed to the high elasticity of the parenchyma which was very

little affected by the acetic acid. Improvement was obtained by freeing the follicles from the parenchyma with needles, under a dissecting microscope, after staining in acetocarmine and before crushing. In this way, the pressure through the coverslip affects the easily ruptured membrane of the follicle directly, and the germinal cells are free to spread.

Another example is offered by the salivary glands of the larvae of a species of *Phoridae* (Diptera), not yet determined, which can be bred easily. The salivary glands of such larvae are structurally different from those of *Chironomus* and *Simulium*, where the gland cells are disposed flatly, around the secretory products; on the other hand they differ from those of *Drosophila*, having a much greater quantity of secretion in the middle of the gland. Smearing of the whole gland gave poor results, owing to the central bulk of secretion, which forms an elastic compact body in the middle of the gland, to the resistance of the external membrane of the gland (which may vary according to the food qualities) and to the tendency of the secretory products to take the carmine dye. An improvement was obtained, by breaking with needles the glands and by extracting the bulk of secretion, during its stay in the acetocarmine, before crushing.

These two cases show that it is necessary to take into due account the structural makeup of the tissues to be smeared, in order to overcome the mechanical difficulties.

ELIMINATION OF FAT AND PROTEIN PRIOR TO THE SELECTIVE STAINING OF BONE

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ABSTRACT.—The technic of staining skeletal systems previously described is often unsatisfactory for fetal specimens of Aves, because of the large amount of fat and protein. The writer avoids this by introducing two preliminary steps: (1) The specimen is placed in equal parts of glycerin, 95% alcohol and distilled water, and 10% aqueous pepsin (with a drop of 6N HCl added) injected into the yolk sac, with 2-3 hours incubation at 40°C. (2) While in 5% aqueous KOH (with a few drops of 2% H₂O₂), the fat areas are injected with cellosolve; and the specimen is left in this solution until skeletal elements become clearly visible. Staining in alizarin red S then follows.

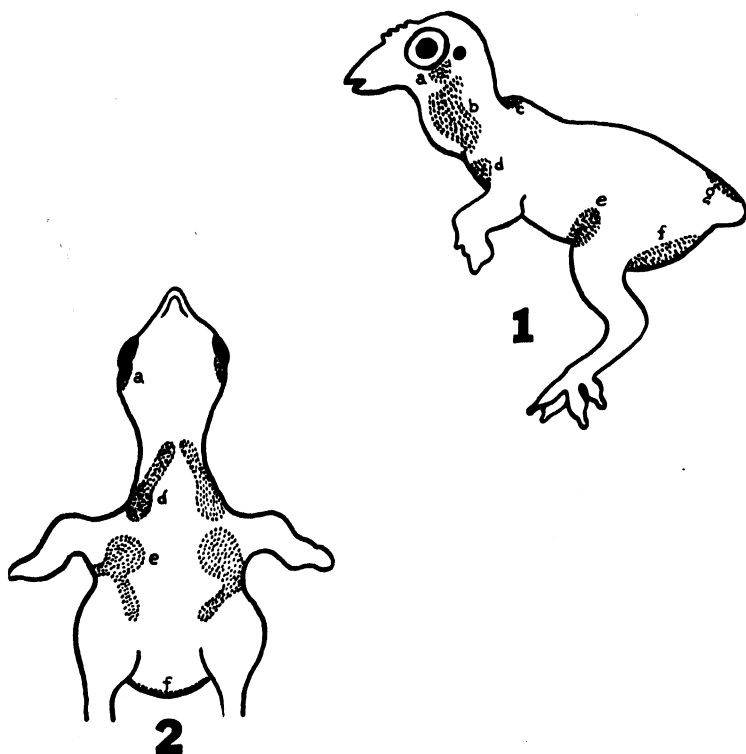
The technic of bone staining employed in the past¹ is perfectly satisfactory for museum preparations, lecture demonstrations, and osteogenic studies insofar as fetal and small adult mammalian specimens are concerned, but in the case of the class Aves there is a great deal of fat which appears as opaque white masses, plus protein in the yolk sac, both of which obscure many of the skeletal elements. A modification is therefore proposed which is specifically recommended for the skeletal elements of the chick, but is also applicable to all vertebrate specimens in a modified form. It has advantages over Dawson's acetone technic (1926) in that it is quicker acting, eliminates excess adipose tissue more satisfactorily, and does not neglect the factor of protein removal.

After conducting numerous controlled experiments with many fat solvents and various forms of proteolytic enzyme digestion over an extended period of time, it has been found that cellosolve (ethylene glycol monoethyl ether), injected subdermally and intra-abdominally after pepsin digestion, works very satisfactorily. The enzyme action must take place before cellosolve treatment since this reagent inhibits the proteolytic action of the acidified pepsin solution.

In the previous technic, no fixative was used prior to saponification with aqueous KOH, but since fresh specimens are not always available, a solution of equal parts glycerin, 95% ethyl alcohol, and dis-

¹True, R. M. (1947.)

tilled water may be employed as a preservative. As Ignalzi (1932) pointed out, alcohol *per se* warps the skeleton in such a way as to demonstrate incorrect anatomical relationships and the aforementioned solution counteracts the dehydrating action of the alcohol yet permits the glycerin's action in fat extraction. Formalin, chromic acid, or any salts of heavy metals should not be employed as fixatives since



FAT DISTRIBUTION CHARTS

21-day Chick

FIG. 1, Lateral View; FIG. 2, Ventral View.

Letters correspond in the two views and show relative distribution of the fat in the superficial and muscular fasciae.

they inhibit the action of the acidified pepsin solution. In the preparation of the pepsin solution, one drop of 6N hydrochloric acid is used to approach the physiological pH. It would appear that this would destroy the minute ossification centers; however, the hydrogen-ion concentration is not increased sufficiently to bring about this de-

structive action. The method works admirably, eliminating the fat deposits, clearing the yolk sac, and presenting a beautifully cleared specimen with the skeletal elements sharply outlined. The proposed method, arranged in the form adopted in STAINING PROCEDURES, is herewith described.

Specially recommended for: 21-day chicks

Designed to: bring out developing bone; eliminate opaque white fat masses; eliminate protein matter in the yolk sac.

Method of fixation: none (or equal parts of glycerin, 95% alcohol, and distilled water)

Preparation of pepsin solution: Pepsin (N.F.), 10 g.; distilled water, 100 ml.; 6N HCl, 1 drop; solution prepared fresh and filtered.

Preparation of staining solution: alizarin red S, (C.C.), 0.001 g.; 2% aqueous KOH, 100 ml.

Staining schedule:

1. Place fresh (or fixed²) specimen in equal parts of glycerin, 95% alcohol, and distilled water and inject the filtered pepsin solution into the yolk sac by means of a standard Leuer syringe and needle. Incubate 2-3 hours at 40°C.
2. Transfer to 5% aqueous KOH to which has been added a few drops of 2% hydrogen peroxide and inject mapped fat areas with cellosolve. Leave in this solution until the skeletal elements are clearly visible through the surrounding tissues. *Note:* If close supervision is impracticable, the specimen may be placed in 2% aqueous KOH (e.g. overnight) and then replaced in the more concentrated potash solution. If the tissues become too soft, harden 12-24 hours in a solution of equal parts glycerin, 95% alcohol, and distilled water, and resume the clearing process.
3. Transfer to dilute (0.0025-0.01%) alizarin red S in 2% aqueous KOH until the desired degree of staining is obtained. The percentage solution varies proportionally with the size of the animal being stained and is greater in adult specimens. *Note:* The use of acid alcohol in the case of overstaining is strongly advised against since the acid solution brings about the decalcification of minute ossification centers. If the dilution of the alizarin is maintained at the proper level, overstaining will not occur.
4. Mapped fat areas may be reinjected with cellosolve at this stage should it prove necessary.
5. Glycerin series (72 hours):
25 parts glycerin, 75 parts 2% aqueous KOH;
50 parts glycerin, 50 parts 2% aqueous KOH;
75 parts glycerin, 25 parts 2% aqueous KOH.
6. Store in glycerin (C.P.) to which a crystal of thymol has been added to prevent mold.

²*Caution:* Specimen should not be fixed in formalin, chromic acid, or salts of heavy metals.

Results: Osseous tissue—red

Muscle tissues—transparent and unstained

Fat deposits—eliminated

Yolk sac—cleared.

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AN ELLIPTOMETER

A SIMPLE METHOD OF MEASURING THE AREAS OF STRUCTURES IN MICROSCOPIC SECTIONS

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ABSTRACT.—A simple method of measuring the cross sectional areas of objects (such as cells) in microscopic sections is described. A beam of light is passed through an adjustable diaphragm and focussed by a lens on a screen of mm. ruled graph paper. The screen may be rotated about a horizontal axis. Adjustment of the aperture of the diaphragm and the plane of the screen yields illuminated areas of variable size and degree of ellipticity. As close a "fit" as possible is made between the illuminated area and the camera lucida tracing of the object to be measured. The lengths of the major and minor axes of the appropriate ellipse are read from the screen. The application of the formula for the area of an ellipse to mean major and minor axes for a group of cells gives the mean cross sectional area for the cell population under investigation.

Few will disagree with the statement that quantitative confirmation of visual impressions in microscopy is to be encouraged. This principle is particularly applicable in the assessment of cytological changes under experimental conditions. The quantitative expression of alterations in cell size, nuclear eccentricity, etc., gives some degree of precision to the observations. Furthermore, quantitative methods are more likely to furnish clues concerning the intracellular dynamics involved in the cytological changes which are observed. Unfortunately, quantitative procedures are tedious and are frequently omitted for this reason. It would seem, therefore, that any procedure which would tend to simplify measurements in microscopic work might have applications in biological research.

The method to be described may be found useful, for example, in measuring the areas of cells in cross section. It is particularly useful in experiments which require the determination of the mean cross sectional area of a large number of cells in control and experimental groups. This information may be used to estimate the mean change in cell area resulting from an experimental procedure. The method is applicable only to large cells. Its accuracy is in proportion to the

degree to which the shape of the cell approaches that of an ellipse. Bearing these limitations in mind the elliptometer may be found preferable, in certain circumstances, to the direct measurement of cell diameters by a filar micrometer eye-piece or to the measurement of areas on camera lucida tracings with a planimeter, the two methods in most common use at the present time. One advantage of the elliptometer, in our experience, has been the relative speed with

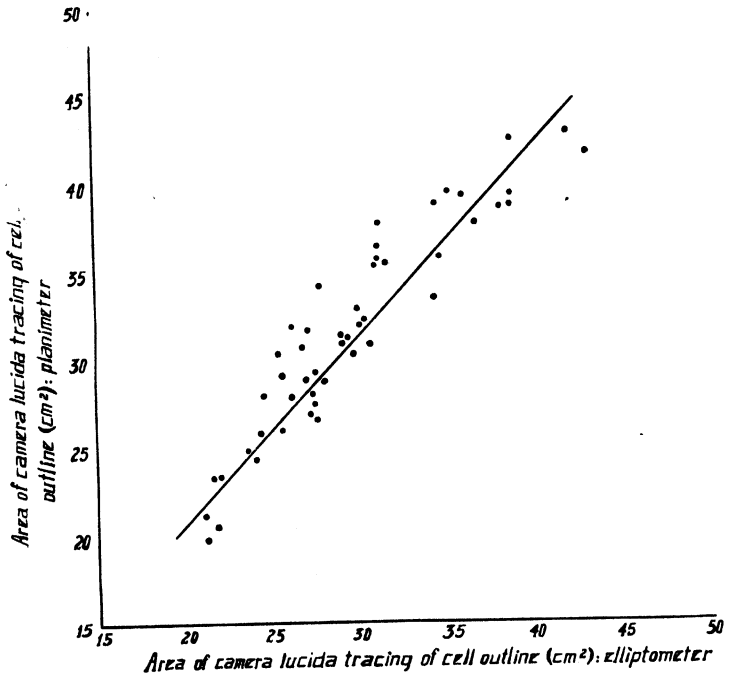
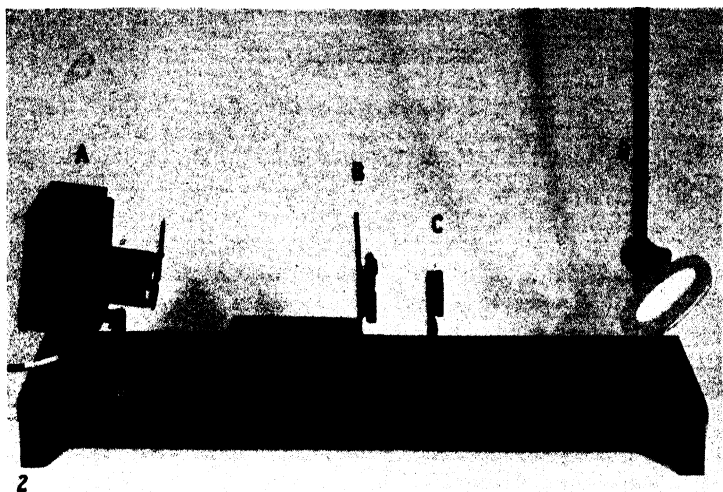
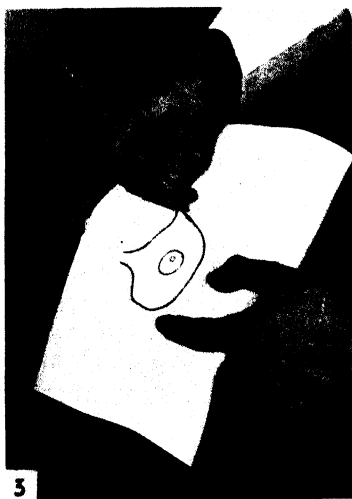


FIG. 1.—The areas of cross sections of fifty motor nerve cells in the spinal cord of the cat were measured by applying the planimeter and the elliptometer methods to camera lucida tracings of the cell outlines. The cells were in a state of axon reaction as a result of peripheral nerve section of 14 days duration. The results of the two methods are in approximately linear relationship. The elliptometer measurements are, on the whole, slightly smaller than the measurements obtained with the planimeter. The measurements refer to the area of the camera lucida tracing of the cell section in cm^2 .

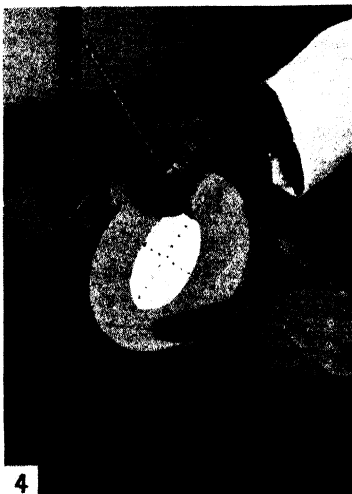
which the measurements may be carried out. Furthermore, since the major and minor axes of the cells are measured as a means of determining the average cross sectional area in a group of cells, information is obtained regarding the ellipticity of the cells in the same operation as that used for the determination of the mean area of the cells in cross section.



2



3



4

FIG. 2.—The elliptometer. (A) the light source; (B) an adjustable diaphragm; (C) lens; (D) screen of graph paper on a board which rotates around a horizontal axis.

FIG. 3.—Method of “fitting” the illuminated area and the camera lucida tracing. The size and ellipticity of the illuminated area are altered by means of the diaphragm and the plane of the screen until the best “fit” is secured. Since the cell outline is very rarely a true ellipse exact “fits” of the illuminated area and the cell outline cannot be obtained. There is, however, a compensatory effect between the amount of the illuminated area outside of the cell outline and the amount of the area inside of the cell outline which is not included in the illuminated area. This compensatory effect gives to the elliptometer method a degree of accuracy equal to other methods for measuring surface areas which are now in common use.

FIG. 4.—Determination of the size of the illuminated area which most closely represents the area of the cell in section. On removal of the camera lucida tracing the length of the major and the minor axes may be read from the ruled screen.

THE INSTRUMENT AND ITS USE

The elliptometer is extremely simple in its construction and application. It consists of a light source (A), an adjustable diaphragm (B), a lens (C) and a paper screen ruled in millimeter squares and adjustable around a horizontal axis (D) (Fig. 2). A good microscope lamp provides a suitable light source. A camera diaphragm will serve to control the diameter of the beam of light which passes through the lens. The latter should be of short focal length. The screen is made by pasting graph paper, ruled in millimeters, on a thin board, the latter being mounted in such a way that it may be rotated about a horizontal axis. An illuminated area appears on the ruled paper. Adjustment of the diaphragm will give areas of various dimensions. Various degrees of ellipticity may be obtained by changing the angle of the screen from the vertical. The apparatus, therefore, provides a simple method of obtaining areas of various sizes and degrees of ellipticity which may be matched with the cells to be studied.

Camera lucida tracings of the cells or other objects to be measured are used. Although direct measurements with the filar micrometer eye-piece eliminate the necessity of making camera lucida tracings, there are advantages in having permanent records of cell body and nuclear outlines. The tracing is laid on the screen and the diaphragm and screen angle adjusted until the best "fit" is obtained (Fig. 3). On removal of the tracing the lengths of the major and minor axes are read directly from the ruled paper (Fig. 4). From these data a figure for the mean major and minor axes for the cell group studied may be obtained. The mean cell cross sectional area may then be determined from the formula for the area of an ellipse (πab , where a is the major semiaxis and b is the minor semiaxis). The same data yield information regarding the mean ellipticity of the cells (b/a). Furthermore the approximate cell center in each cell may be determined on the camera lucida drawing by marking the midpoint between the ends of the major axis, the latter points having been recorded on the tracing when the elliptometer measurement was being made. The cell center is a useful reference point in assessing such factors as nuclear eccentricity. More extensive statistical analyses of the data may be made when indicated.

The elliptometer has been used by us to measure the cross sectional areas of over 2000 motor nerve cells while investigating certain changes which these cells undergo as a result of axon section (to be published). Following the usual practice in such work, measurements were limited to cell sections which contained the nucleolus.

Exact "fits" of the illuminated area and the camera lucida tracings were difficult to achieve since motor neurons are very irregular in outline as a result of the processes given off by the cell body. A similar difficulty is encountered, however, in planimeter measurements since, in the latter method, an arbitrary line must be established between the cell body and the cell processes. Fig. 1 illustrates the degree of correlation between planimeter and elliptometer measurements of the cross sectional areas of each of fifty ventral horn cells in a state of axon reaction 14 days following section of the sciatic nerve. The relationship is roughly linear with the elliptometer measurements, on the whole, slightly less than the measurements obtained with the planimeter. As a result, the curves obtained separately by planimeter and elliptometer methods for the change in the size of the cell body at various intervals after peripheral nerve section are so similar that there is little to choose between them as regards accuracy. In these circumstances the elliptometer method is the one of choice since it is rapid and since information regarding the lengths of the major and the minor axes of the cells may be obtained in the course of measuring cell areas. The determination of the central point in individual cells was found useful as a reference point in measuring the degree of nuclear eccentricity at various stages of axon reaction.

The elliptometer, then, is a simple instrument for the quantitative study of areas in microscopy. It is felt that others may find the method useful in various fields of biological research.

It is a pleasure to acknowledge our indebtedness to Dr. A. C. Burton, Associate Professor of Physiology, for his advice regarding quantitative methods in histology. The apparatus was assembled by Mr. C. Jarvis, technician in the Department of Anatomy. The photographs were prepared by Mr. W. J. T. Austin, Art Service, University of Western Ontario. This work was carried out while the authors held a grant-in-aid from the Ontario Cancer Treatment and Research Foundation.

APPLICATION OF PECTIC ENZYMES TO MACERATION OF PLANT TISSUES FOR MICROSCOPIC STUDY

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ABSTRACT.—A new method of maceration of relatively soft plant parts, such as petioles, fruits, and fruit skins, which depends upon the treatment of the material with pectic enzyme solutions is described. The commercial preparation Pectinol W, (manufactured by Rohm and Haas Co., Washington Square, Philadelphia 5, Pa.) as well as pectic enzymes secreted by growing *Aspergillus* species upon pectin-containing media were effective in macerating a variety of plant materials.

Maceration technics have long been in use for the study of cell wall structure of many types of cells. Most of these methods, such as Jeffrey's (see Johansen, 1940) employ very strong chemical solutions, and may only be applied to relatively tough woody tissues. Kisser (1926) described more gentle chemical technics for accomplishing maceration without adversely affecting the cell contents. Foster (1944) found Priestley's (1938) method, which depends upon chemical solution of intercellular pectic substances by acid alcohol followed by ammonium oxalate, to be useful for the maceration and separation of the complex ramified sclereids of the *Camellia japonica* petiole. Since commercial preparations of pectic enzymes of microbial origin are now commonly used for the removal of pectic substances in clarification of fruit juices and in other applications by the food processing industry, it seemed probable that such enzymes might be useful in maceration of plant tissues for microscopic study. [See Phaff and Joslyn (1947) for a comprehensive review of pectic enzymes]. The well known effect of common molds in breaking down fruit and vegetable tissues to a soft mass, was a further suggestive observation.

The technic was tested on a variety of plant tissues—including petioles of *Camellia japonica* (Fig. 1), seed coats of succulent fresh or frozen peas (*Pisum sativum*) (Fig. 2), skins of the fruits of prunes (*Prunus domestica*), apricots (*Prunus armeniaca*), and figs (*Ficus carica*), as well as the flesh of pear (*Pyrus communis*) and apricot fruits.

The technic consists of placing a small piece of the tissue into a 1–5% solution of "Pectinol W" (Rohm and Haas) in water or sodium acetate, acetic acid buffer at pH 5, in a small vial with 0.05 to 0.1%

merthiolate crystals added to inhibit microbial action, deaerating in an aspirator, and allowing this to remain overnight at 28 to 30°C. The major portions of all these tissues were successfully macerated by this treatment. A gentle tearing apart with needles on a slide was generally sufficient to spread the cells apart so that they could be readily studied. A period of 15 to 24 hours was usually a sufficient length of time, and for most materials a 1% solution of the enzyme was of suitable strength. While an aqueous solution of the enzyme was effective in the maceration of the tissues, the buffer solution



FIG. 1.—Sclereid from *Camellia japonica* petiole macerated with pectinol "W". 100X.



FIG. 2.—Sclereids from seed coat of *Pisum sativum*, macerated with pectinol "W". 250X.

was preferable since the optimum pH of the enzyme is approximately 5. Toluene has frequently been used to control microbial contamination in enzyme preparations, but merthiolate was found more effective since toluene sometimes allowed bacterial development, especially when pea skins were being macerated with aqueous solutions of pectinol. Some of the plant materials tested, such as the uppermost epidermal layers of the peeled apricot and prune skins,

were not readily dissociated by the enzyme preparation. This may be because these particular portions did not have pectic intercellular substances, or the enzyme preparation may not have contained the proper combination of pectolytic components, or there may have been imperfect penetration of the enzymes into these layers.

In addition to the commercial enzyme preparation, several other preparations of mold enzymes, made by Mr. M. Ibarra in this laboratory, were tested for their effectiveness in maceration of these tissues. The enzymes were prepared by growing *Aspergillus awamori* (U. S. Dept. Agr. Northern Regional Research Laboratory No. A 1947), or *A. carbonarius* (N.R.R.L. No. 369) on about one liter of medium containing citrus pectin, in a 3 to 4 liter Erlenmeyer flask, at room temperature for about 20 days, and filtering off the culture medium, which contained the enzyme. For these tests, the enzyme extracts from both of these molds were quite effective, and appeared to give even better, cleaner cut results than did the commercial preparation. Similar enzyme extracts prepared from other molds such as *Penicillium chrysogenum* (discussed by Phaff, 1947) and other species may be equally effective when applied to this procedure.

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A TRI-BASIC-DYE STAIN FOR NERVE CELLS

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ABSTRACT.—A new staining method has been developed for the study of nerve cells and Nissl granules which combines three basic dyes, cresylecht violet, toluidine blue and thionin. The use of this tri-basic-dye stain results in finished preparations that are critically stained and permanent. Paraffin sections (4 μ sections preferably) are mounted on slides by the starch medium, deparaffinized and stained by the tribasic staining solution. After differentiation in acidified distilled water, sections are dehydrated, returned to stain solution and again dehydrated, then cleared and mounted in Clarite. Various vertebrate material including normal and pathological human tissues have been stained with this triple dye solution. Especially for pathological material, re-immersion of slides in the staining and 80% alcohol solutions before mounting, differentially intensifies the staining reaction. Fixatives used were 10% formalin, 95% alcohol, Bouin and formalin-Bouin (10% formalin followed by Bouin).

INTRODUCTION

Cresylecht violet, toluidine blue and thionin have been used singly for staining nerve tissues. Cresylecht violet has long been employed for its metachromatic properties in making permanent preparations of nervous tissue; it stains nuclei violet, protoplasm blue and various granules either red or violet. Toluidine blue is a widely used nuclear stain and also demonstrates especially well Nissl granules and other chromophilic bodies. Thionin has a very pronounced metachromatic effect and stains tissues from blue to reddish-violet. It has been recommended by Bean (1927) for the staining of Nissl granules. Haynes (1927) and others have investigated the staining properties of thiazin dyes used separately as biological stains.

In the course of an investigation of elements of the central and autonomic nervous systems, particularly of the innervation of the epithelial cells of the gastric mucosa (Spoerri, 1947), a staining solution was developed which combines the above mentioned three basic dyes. Neurons stand out distinctly on a pale background and the processes can be followed for a considerable distance. The cytons themselves stain dark purple emphasizing the blue tint while the dendrite and axon processes and endings present a somewhat lighter

shade, bluish to reddish. Granules in the cell body as well as in the protoplasmic processes appear purple or reddish. Nuclei and nucleoli are well differentiated.

This color discrimination has not been obtained with cresylecht violet alone which the author considers the main dye. The advantages of the tribasic combination method over the use of single dyes may be explained by the simultaneous reaction of the three basic dyes. This not only adds to the staining intensity of the final preparation but also appears to increase the affinity of the tissue to the main dye.

Fixatives used were 10% formalin, 95% alcohol, Bouin and formalin-Bouin (10% formalin followed by Bouin). Normal and pathological tissues of the central nervous and digestive systems of the guinea pig, rabbit, cat, dog, monkey and human have been stained by this tribasic method.

Preparation of Tri-basic-dye Solution

Cresylecht violet	2 grams
Toluidine blue	1 gram
Thionin	0.5 gram
30% ethyl alcohol	200 ml.

The solution is filtered before using it the first time, but need not be filtered again on further use. The dyes used were manufactured by G. Grüber & Co., Leipzig. American dyes (cresylecht violet, toluidine blue and thionin) sold by the Coleman & Bell Co., Commission Certified and noncertified, have given equally satisfactory staining results with the lots of stains used.

METHOD

1. Mount paraffin sections (preferably 4μ) on slides by the starch medium (Spoerri, 1939) and pass through xylene, absolute alcohol, 95% alcohol and 80% alcohol.
2. Dip slides into staining solution which has been heated in a beaker to $80-90^{\circ}\text{C}$ 5-10 sec.
3. Pass slides for primary differentiation through acidified distilled water (10 drops of concentrated H_2SO_4 or HNO_3 in 200 ml. of distilled H_2O) 1 sec.
4. Dip and agitate slides in distilled water 1 sec.
5. Differentiate further in 80% and 95% alcohol 1-2 sec. each
6. Return to 80% alcohol 1 sec.
7. Dip and agitate slides in the still warm tri-basic stain solution 1-2 sec.
(Repetition of steps 6 and 7 will intensify the result)
8. Rinse in distilled water

9. Dehydrate in 80%, 95% and 100% alcohol.....1 sec. each
10. Xylene.....1 min.
11. Xylene.....3 min.

DISCUSSION

Most types of Nissl stains utilize aqueous dye solutions followed by differentiation in acid alcohol. Preparations, resulting from the use of the tri-basic-dye solution proposed by the author, are permanent and present clear differentiation of the nerve cell, when the staining solution has an alcoholic medium and differentiation is accomplished in acidified water and alcohol, as indicated in steps 3-6 of the procedure. Further intensification of stained components is achieved through a second application of the staining solution (step 7).

This tri-basic-dye staining procedure has the advantage of being short, in addition to giving differentially stained and permanent preparations. Preparations kept by the author for five years and longer show no evidence of fading and are in use for teaching histology and neuroanatomy. The intense light used in photography has had no effect on the stain.

Formalin-fixed preparations give clear-cut staining results with less shrinkage of cells than does alcohol-fixed material. The combination fixative of formalin and Bouin is especially valuable for human autopsy and pathological material. Several repetitions of steps 6 and 7 have been found particularly advantageous for the differential staining of pathological material.

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TRIPHENYLTETRAZOLIUM CHLORIDE, A VALUABLE REAGENT IN STAIN TECHNOLOGY¹

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ABSTRACT.—Triphenyltetrazolium chloride is proposed as a reagent in studies of cellular physiology. It has been employed to locate intracellular sites of dehydrogenase activity in sugar cane (*Saccharum officinarum*) and as a valuable tool in correlating the action of antibiotics with inhibition of dehydrogenase systems.

Triphenyltetrazolium chloride has been proposed as a reagent to test the viability of seeds (Lakon, 1942a,b; Cottrell, 1947; Porter, Durrell, and Romm, 1947) and of cuttings of trees and shrubs (Vaugh 1948). The basis of the reaction is reduction of the soluble colorless triphenyltetrazolium salt to the insoluble red formazan². Therefore, vitality is estimated in terms of reducing activity of the embryonic tissue and depends upon a principle enunciated long ago by Lavoisier, namely, that ability to rehydrogenate oxides is an eminent property of living tissues. Since this property is not peculiar to seeds, the reagent might be expected to discriminate also between other tissues or cells with high reducing activity and those with low or no such capacity.

Experimentally, we have found this surmise to be correct. The salt has proved to be valuable as a reagent for locating intracellular sites of dehydrogenase activity in sugar cane and especially valuable in bacteriological researches on the mechanism of action of penicillin and other antibiotics. These uses suggest numerous other applications.

EXPERIMENTAL

*Triphenyltetrazolium chloride*³ as an histological reagent. When the

¹This work was supported in part by a generous research grant from the Cutter Laboratories, Berkeley, California.

²The chemistry of the tetrazolium salts has been thoroughly reviewed by Benson (1947).

³We wish to acknowledge with thanks the courtesy of Dr. F. R. Benson, Remington Rand, Inc., South Norwalk, Connecticut, and of the research group at Pennsylvania State College, both of whom supplied us with research samples. Under the trade name, Vitastain, 2,3,5-triphenyltetrazolium chloride is now available commercially from Arapahoe Chemicals, Inc., 2800 Pearl Street, Boulder, Colorado.

base of a freshly cut stalk of sugar cane (*Saccharum officinarum*) is immersed in a 0.5% aqueous solution of 2,3,5-triphenyltetrazolium chloride, the soluble uncolored reagent is absorbed and transported rapidly to the meristematic regions of the buds where it becomes reduced to the insoluble red formazan. Similarly, reduction and consequent deposition of the red precipitate occur in the meristematic tissue about each of the upper nodes, immediately beneath the apical bud; these nodal tissues have been shown by one of us (Dufrenoy, 1942) to give a strong positive reaction for pyridoxine and are known to be sites of high metabolic activity.

Microscopic examination of freehand longitudinal sections of unfixed tissues of the stalk reveals that reduction of the tetrazolium salt also occurs about the pores of the sieve tubes and about other intercellular connections; the cytoplasm surrounding which becomes conspicuously stained by the red formazan. Sugar cane tissues are remarkable in that they contain all intermediates between the most highly differentiated types of intercellular contacts, found in sieve-plates at the upper and lower ends of the sieve-tubes of the phloem, and simpler modes of intercellular contacts found in the lateral walls of the sieve-tubes or of other elements of the phloem and in the walls of parenchymatous cells. These intercellular devices for allowing the cytoplasm of one cell to come into close contact with that of adjacent cells have been minutely described by Mangenot (1926) under the name of synapts. The sites of reduction of the reagent are shown in Fig. 1. This figure shows at the right a longitudinal section through the phloem of a cane, the base of which had been immersed 48 hours in a 0.5% solution of 2,3,5-triphenyltetrazolium chloride: the synapts (*sy*) which were actually colored bright red with the insoluble formazan derivative of the tetrazolium salt are featured solid black in the drawing. At the extreme right of the section is a sieve plate, showing the deposition of formazan about the pores. The drawing at the left in Fig. 1 shows part of a freehand longitudinal section of living parenchyma of a cane, the base of which had been immersed in an aqueous *M*/1000 solution of sodium selenite. Focussing on the plane of the lower wall of the central cell brings into evidence the pits which appear as "crossed spindles" and are outlined by the deposits of metallic selenium (actually bright red, but featured black in the drawing); focussing on the side walls reveals the pits, or synapts (*sy*), also outlined by deposits of selenium (*se*). Further microscopic study showed that the sites of reduction generally coincided with the location of oil droplets whether in the cytoplasm near the plasmodesmata (synapts) or between the starch

grains within the amyloplasts, (a) in Fig. 1. Identical results are obtained when the tetrazolium salt is used in place of the selenite which, incidentally, antedated by some years the tertazolium salts as physiological reagents (Dufrenoy, 1929; Eidmann, 1938; Eggebrecht and Bethmann, 1939; Lakon, 1940).

Triphenyltetrazolium chloride as a bacteriological reagent. If at the conclusion of incubation, standard penicillin assay plates are flooded with a 0.5% solution of 2,3,5-triphenyltetrazolium chloride, the zones

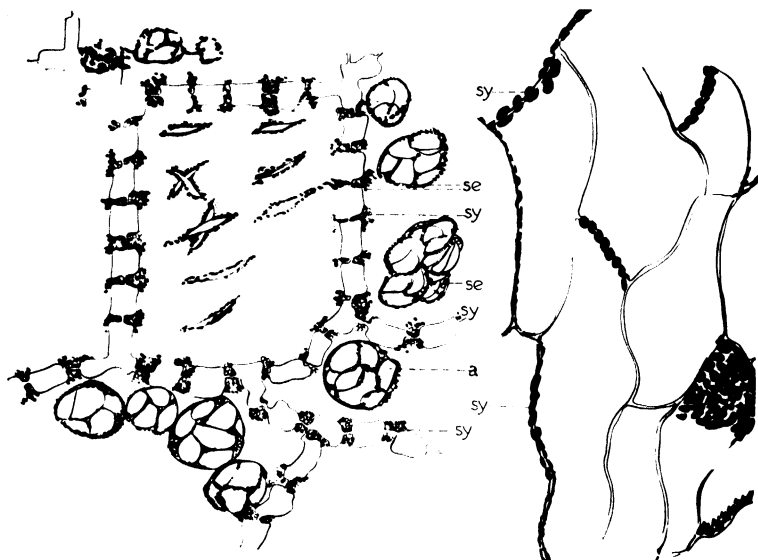


FIG. 1.—Freehand sections of tissues of sugar cane.

Right. Section through phloem of cane, base of which was immersed in solution of 2, 3, 5-triphenyltetrazolium chloride.

Left. Section through parenchyma of cane immersed in M/1000 solution of sodium selenite. The deposits of red formazan and metallic selenium, respectively, are represented as solid black. (See text for further explanation.)

of inhibition are promptly outlined by a bright red ring. The inhibition zones themselves are uncolored and the general uninhibited background becomes faintly pink, gradually turning red over a period of an hour or two. This indicates lack of, or very low, reducing activity in the inhibition zones, each of which is surrounded by a circumjacent area of extremely high reducing activity which corresponds with the ring of enhanced growth that is a constant feature of such assay plates (Dufrenoy and Pratt, 1947a,b; Pratt and Dufrenoy, 1947a,b, 1948). The faint pink color of the other portions of

the plates indicates that there is only moderate reducing activity in the organisms that have been unaffected by penicillin. Unseeded control plates exposed to penicillin in the same way show no reaction with the tetrazolium salt. If, however, the seeded and incubated plates are treated for a few minutes with a 1% solution of formaldehyde to inactivate the dehydrogenase systems before the tetrazolium salt is added, no color change occurs in any part of the plate.

A practical application of tetrazolium chloride in penicillin assays is seen in the development of a rapid assay that decreases the time required to complete a test from 16 or 18 hours to three hours or less, and that provides a linear calibration curve over the range from 1 to 8 units/ml. This may be done according to the methods described for other dyes (Pratt and Dufrenoy, 1947c).

CONCLUSION

The introduction of 2,3,5-triphenyltetrazolium chloride in stain technology makes it possible to detect histochemically the sites of dehydrogenase activity with a precision heretofore unattainable, and to correlate the dehydrogenase activity with the apparent redox potential of the triphenyltetrazolium chloride \rightleftharpoons formazan equilibrium which has been reported to be poised about -0.08 volts (Mattson, Jensen, and Dutcher, 1947). This system operates in a physiological range, since glucose dehydrogenase-coenzyme I, in the presence of its substrate, has been reported to reduce the tetrazolium salts at pH 6.6.

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CLEARING BRAIN TISSUE WITH ANETHOLE

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Groat (1941) called attention to the necessity of matching the refractive index of the tissue and clearer to obtain maximum transparency of *in toto* cleared specimens. Tissues that are refractively almost homogeneous, such as muscle, can be cleared after bleaching and dehydrating by placing in an oil of similar refractive index. Brain tissue, on the other hand, is composed of lipids and protein which have different refractive indices. Since protein forms the framework of the brain, it is, therefore, necessary to remove the lipids in order to have a tissue of a relatively uniform refractive index. The refractive index of brain tissue dehydrated in alcohol and extracted with ether, as determined on an Abbé refractometer, is 1.560.

The lipid composition of brain tissue according to Koch (1909), as quoted in Mathews (1939), is as follows: phospholipids—27.3% of dry matter; cerebrosides—13.6% of dry matter; cholesterol—10.9% of dry matter. The phospholipids according to Hill & Kelly (1943) are composed mainly of: lecithin—48 p.p.m., soluble in ether and in alcohol; cephalin—200 p.p.m., soluble in ether, insoluble in alcohol; sphingomyelin—56 p.p.m., insoluble in ether, soluble in hot alcohol. These are all insoluble in acetone. The cerebrosides are soluble in hot alcohol, insoluble in ether. Cholesterol can be extracted with ether and alcohol. (The data on solubilities are taken from Harrow, 1943.)

Therefore, formalin fixed brain tissue may be dehydrated by using the alcohol series starting at 35%, or higher if one wishes to risk more shrinkage, and changing every few hours depending upon the size of the tissue. The tissue is then placed in 100% alcohol for 1–2 days and kept at 60° C. by placing in an incubator or by other means. Frequent changes of solution are advisable. The remaining lipid is then removed by ether. A piece of tissue of 1 cm. in thickness will be satisfactorily extracted if left in ether for 2 days after the alcohol series. Frequent changes of solution are again advisable.

By now placing the tissue in an oil of very similar refractive index it will soon become transparent when brightly illuminated. Anethole ($C_{10}H_{12}O$) was found to be the oil of choice. It has a refractive index of 1.5614, is colorless, very slightly soluble in water, soluble in alcohol and ether, and is liquid at ordinary temperatures. It can be obtained

from the Eastman Kodak Company, Rochester, New York for about \$.85 per 100 gm.

After the tissue has been placed in anethole it is advisable to leave the container uncovered so that the displaced ether may escape.

The transparency of the tissue depends upon the completeness of lipid extraction. Larger pieces of tissue such as $4 \times 6 \times 3$ cm. must be kept in warm 100% alcohol for a week, and in ether for 1-2 weeks. Even then, the entire block of tissue will appear only translucent when cleared in anethole. However, sections of $\frac{1}{2}$ to 1 cm. in thickness cut from this large piece will be cleared sufficiently to enable one to study injected vessels, etc. under the binocular with good illumination. It is probable that a large piece of tissue could be cleared satisfactorily if the alcohol and ether extraction were carried out by means of an intermittent siphoning distillation extractor.

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THE STAINING OF RADULAE

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ABSTRACT.—A review of the various methods of staining and mounting radulae is given. Normally the radula should be extracted with 0.5 to 1% sodium hydroxide solution, and the associated tissues removed before staining. Two staining methods are recommended for facilitating the interpretation of radulae. Newly formed teeth and the bases of older ones are well stained by saturated aqueous chlorazol black E (up to 10 minutes). A more uniformly stained specimen, in which the cusps of all but the young teeth are alone stained, may be obtained by using the "oxidation-dahlia technic". The radula is oxidized in N/10 potassium permanganate solution until black and subsequently decolorized in saturated aqueous oxalic acid. It is then stained in 0.1% aqueous dahlia (Hofmann's violet), the staining time varying from 10 to 30 minutes, according to the material. It is then dehydrated and passed through xylene and clove oil into Canada balsam. Other mountants may be employed but glycerin jelly is only recommended for the rapid examination of unstained radulae. Several other staining methods are mentioned, and general precautions to be observed while mounting are discussed.

Although radulae, the chief components of the feeding mechanisms of all head-bearing molluscs (with but few exceptions) are of very great taxonomic value, their mounting is not discussed in the standard works of microtechnic. The mounting of radulae was very popular during the 19th century, particularly as "polariscope objects", but one very rarely meets any old slides of stained radulae.

The radula may be obtained from a recently killed mollusc or it may only be available in a completely dried out animal or one preserved in alcohol. Radulae which have been preserved in alcohol, and which have later dried out, are very brittle and extreme care must be taken when handling them. Such radulae are frequently brown enough not to need staining, and thus excessive handling can be avoided. In the case of medium to large animals the radula can be extracted by dissection and subjected to treatment, but in the case of small snails the whole body may be treated. The best results are obtained with fresh material. The following account of the preliminary extraction treatment is mainly based on notes by Lt. Col. Peile who is undoubtedly the most skilled British mounter in this

field. Aqueous sodium or potassium hydroxide (0.5 to 1.0%) is the most usual extraction reagent. Good results are obtained by prolonged treatment of the snail or odontophore (i.e., radula plus surrounding tissues) in the cold reagent. For rapid results, however, warming is essential and with tough specimens boiling is necessary. If the radula is to be used for delicate staining experiments, caustic treatment must not be employed. The snail should be allowed to decay in water until quite soft, treated with alcohol to remove the smell, and the radula dissected out. A binocular dissecting microscope is almost necessary for all save the largest material. Having secured the radula it is important to remove certain membranes which are attached to its outer margins. A large radula will stand a lot of rough treatment with needles and brushes. The small ones may be safely handled with match sticks sharpened to the very finest of points. Care must be taken, to see that the teeth are not clogged with particles of vegetable matter and grit. Such particles are not infrequently left undissolved by the caustic. This particularly applies to the freshwater *Taenioglossa*; in the marine genus, *Strombus*, the radula is often completely clogged with matter. Such clogging material seriously interferes with staining technics. Washing in several changes of distilled water is of value, and with a large radula a good deal can be removed with needles. When clean and freed from membranous matter the radula is ready for mounting.

The old method of mounting radulae was to mount them unstained, directly into glycerin jelly. This was favored by the serious students since it was a quick method. The Rev. Prof. Gwatkin of Cambridge (England), who probably mounted more radulae than any other person, invariably used it. Although it is undoubtedly a very useful mountant for a rapid examination, it has, owing to its low refractive index, optical properties quite unsuited for accurate work. Glycerin after a long time allows the radula to oxidize to a brown color and it ceases to be anisotropic. Radulae meant for "polariscope slides" were therefore mounted in Canada balsam, but this renders the radula almost invisible and is not of much value for normal visual work. Euparal is a useful mountant and may be used after dehydration in any of the usual dehydrants, alcohol, dioxane, acetone etc. The writer has recently found a direct mountant ("Wallerite") invented by Mr. Waller of London to be of great value for the rapid examination of radulae. This material, which hardens to a brittle resin, contains acetic acid and thus helps soften the radula for manipulation. It is superior to glycerin jelly, but resembles it in that radulae may be directly mounted in it from water (after blotting off

excess) or acetic acid. For accurate work, however, the optically sound method is to stain the radula and use a mountant with a refractive index close to that of the radula itself, Canada balsam and polystyrene being undoubtedly the best. These mountants have refractive indices close to that of glass and the full resolving power of the microscope is thus utilized. Photographs comparing radulae mounted in glycerin jelly with those stained and mounted in balsam are given by *Bowell (1915, 1924a)* and *Verdcourt (1948)*.

The radula is divided into distinct transverse areas which have widely differing chemical properties. First, there are newly formed young teeth at the extreme posterior end (nascent end). These later become fully formed and harder and eventually of a different nature entirely. *Bowell (1915, 1924a and b)* states that at this point a layer of calcium-containing enamel is secreted on to each tooth (=uncus). *Pantin and Rogers (1925)*, discussing the radula of *Buccinum*, term the material forming the young teeth chitin A and that forming the older teeth chitin B. They proved that the latter was an amphoteric substance having an isoelectric point at about pH 2.6, and that a certain amount of chitin A persisted at the base of every tooth. It will be noted that these two ideas are basically the same, though it is probable that enamel is not laid down but that the chitin on the upper part of the older teeth is hardened and modified by a secretion. New teeth are continually laid down and each row goes through these stages being finally worn away when it comes to the anterior end of the radula. A general account of the morphology will be found in any advanced textbook of zoology.

Acid dyes only stain the young teeth, and chlorazol black E and chlorazol black BHS are very good for this. It is a mistake to restrict study to the older teeth since the young ones often show features which are later worn down. The extracted radula is placed in a saturated aqueous solution of the dye and examined at intervals, being removed when adequately stained, dehydrated and mounted in the usual manner. The basal membranes stain readily throughout the length of the radula and it is important not to overstain. Alcoholic solutions are more rapid, that in methyl alcohol being extraordinarily so. It is impossible to specify a staining time since this varies from $\frac{1}{2}$ minute to 10 minutes and must be determined by trial. This statement applies to all radula staining methods. Results are figured by *Verdcourt (1946)*. Other acid dyes will produce a comparable result. Bordeaux red is good and may be used in conjunction with chrysoidin as a double stain. Acid dyes are useful for staining radulae in serial sections of molluscs.

Uniform staining of whole radulae by means of acid dyes is possible if certain drastic measures are adopted. Boiling with acids, e.g. glacial acetic, often works. T. Rogers (1924) devised an electrical method. The radula is extracted by long boiling with 10% sodium hydroxide, followed by 10% acetic acid washing. It is then placed absolutely flat between damp filter papers laid on a plane zinc plate, flooded with dye and a similar copper plate adjusted on top. An E.M.F. of 2 volts is then applied between the two plates. The time required is again variable. The radula is then washed with 25% HCl-alcohol until the stain is completely removed from the basal membrane, dehydrated and mounted. Saturated solutions of indigo carmine, magenta, carmine, and VanGieson's in 33% acetic acid are prepared; they are used saturated for land species and diluted for marine species.

Bowell found that a saturated aqueous solution of chrysoidin would stain the 'enamel' direct without any preliminary treatment. The time required may be as long as 1 hour. The staining is quite good and photographs may be taken with the aid of a filter, but it does not compare with the "dahlia" method. Other dye derivatives of p-amino-azo-benzene could probably be utilized.

The "oxidation-Dahlia technic" which is capable of yielding very beautiful results was worked out by E. W. Bowell. The radula is first warmed or boiled with acidified potassium permanganate until it is quite black. The actual strength of the permanganate is not critical but *N/10* is suitable. A drop of glacial acetic acid should be added to every 4 or 5 ml. before use. When the radula is quite black (it is instructive to mount one at this stage) it is placed in a saturated solution of oxalic acid to decolorize, and subsequently washed. Almost any basic dye may now be employed. Bowell recommended dahlia,¹ and gentian violet gives very similar results. Magenta gives a rather pale color. These dyes may be used in 0.1% aqueous solutions. Hematoxylin may also be recommended and can sometimes be used without oxidation on fluvial species. After staining, the radula is dehydrated, passed through xylene, and if desired through clove oil, into balsam or styrene. Staining time is as usual variable, but 30 minutes is about the maximum required.

No matter how the radula is mounted certain points must be observed. It must be mounted the right side up, i.e. teeth uppermost, and preferably in a standard direction. Experience is needed for this, and it is useful to note that in most cases when a radula is the

¹The "dahlia" of Bowell was a German product of uncertain identity. The present writer employs a "dahlia violet" of the British Drug Houses, stated in their catalog to be synonymous with Hofmann's violet, C.I. No. 679.

right way up the basal membrane bends down anteriorly and up posteriorly (i.e. at the nascent end). If the radula is dehydrated on the slide, it is often advantageous to remove the excess of moisture with a blotter (e.g. a cigarette paper). If the radula should happen to be smooth side uppermost it will adhere to the paper. Many microscopists will probably prefer to use the more orthodox method of dehydrating in a dish, but for very minute radulae the whole process including extraction must be done on one slide (Verdcourt, 1946).

From the point of view of beauty it is pleasing to mount a whole radula, but useful information may be obtained by examining isolated teeth especially if some of these are lying on their sides. A radula is stained by the dahlia technic in the usual manner but when placed in balsam it is violently teased out with needles until a fair proportion of isolated teeth are obtained.

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AN IMPROVED METHOD OF DECALCIFICATION

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ABSTRACT.—Following several experimental investigations, an improved method of decalcification has been devised. The principle of this decalcification method is to obtain complete decalcification by a mixture of as high pH as possible without diminishing the stainability of the Nissl-granules (with Einarson's progressive staining method by means of gallocyanin). This is accomplished by the help of a buffer solution of equal parts of 8 *N* formic acid and 1 *N* sodium formate (pH 2.2). After-treatment consists only in rinsing in flowing water for 24 hours. Dehydration is in alcohol (70%, 96%, 100%); cedar oil; ligroin. Embedding in paraffin follows.

After some years in experimental histological research on the internal ear of guinea-pigs it has become clear that the histologic technic of this special field is somewhat deficient, owing partly to the situation of the internal ear, enclosed in bone, and partly to the extreme sensitivity of the internal ear to outer influences.

As late as 1940 this deficiency was pointed out by Werner in his comprehensive book about the labyrinth. In this connection, Werner, also wrote that a closer study of fixation, decalcification and embedding was necessary. In other fields Lillie emphasized that quick and satisfactory decalcification and satisfactory staining of the bone marrow still offered a technical problem.

The most important difficulty was to obtain a selective, progressive staining of the Nissl-substance (by means of Einarson's gallocyanin) in the internal ear ganglia after the tissue had been submitted to the decalcification which was necessary for sectioning the preparations.

In most histologic manuals several decalcification methods are summed up, but only very few and inextensive studies have been performed to find out what the principle of decalcification is and which method is preferable.

Schaffer (1902) tried to bring order from the confusion by means of a systematic testing of the then known decalcification methods. He compared decalcification time to swelling or shrinking of tendon and bone tissues. After this, the author recommended decalcification in 5% aqueous solution of nitric acid—a method which is still the most widely used.

The first progress in decalcification methods since the work of

Schaffer was achieved by Evans and Krajian (1930), when they published a new method of decalcification. The decalcification agent was composed of equal parts aqueous 85% formic acid and 20% aqueous sodium citrate. In comparison with the effect of nitric acid, the authors found that the cellular elements were practically unaffected (like the tissues not subjected to decalcification), and staining was perfect. No mention was made of the relation between decalcification time of treatment in the acids mentioned.

In regard to decalcification of the bone marrow, Lillie (1944) published a comprehensive study on various decalcification agents and their relation to the Romanowsky staining. He was of the opinion that a control of the injury of bone marrow staining would be possible through control of the initial pH. He therefore made a series of buffered mixtures of sulfurous acid and formic acid for comparison with nitric acid, trichloroacetic acid and acetic acid. A 5% aqueous solution of formic acid was found the most satisfactory.

The problem of decalcification is that tissues containing bone cannot commonly be used for sections and the method of grinding sections is excluded for cytologic purposes. In some way one must alter the consistency, as the tissues, in general, soften. This only can be done by extraction of the inorganic bone components. This again is to say that the bone-containing tissue must be submitted to an acid action inasmuch as enzymatic procedures can be excluded in cytologic researches. But this acid action also influences the organic tissue components. This influence must be studied partly in stained sections, partly in unstained sections. By means of the stained sections alterations of stainability caused by the acids can be studied. In the unstained sections it is possible by help of dark-field microscope, fluorescence microscope, or phase-contrast microscope, combined with photographing, to ascertain alterations of protoplasm or cells caused by swelling or shrinking etc.

In order to try to find a method of decalcification unusually gentle in action, without too much reduction of decalcification power, a systematic, comparative investigation was undertaken of a number of known decalcification agents, either pure or mixed with various substances, in order to reduce the tissue injury of the acids.

By help of specially prepared bone and nucleus tissue blocks, it was found in all trials that 1 N and 2 N formic acid gave relatively the best nuclear staining at a not too long decalcification time.

Following investigations on formic acid of various strengths, it proved that 4 N formic acid, of pH 1.1, gave the fastest decalcification but with a somewhat impaired nuclear staining, compared to

formic acid of lower normality. Formic acid of higher normality showed a greater tissue injuring action and at the same time the decalcification proceeded more slowly, especially in the case of concentrated formic acid, which decalcified so slowly that the tissue was destroyed.

As by another trial a certain parallelism was ascertained between the pH of the decalcifying agent on one hand and decalcifying time and nuclear staining on the other, so that a low pH gave quick decalcification but inferior nuclear staining and vice versa. Furthermore one did not like to use an unusually low pH for the most common fixatives. It therefore was quite pertinent to try to preserve the decalcifying effect of formic acid with simultaneous diminution of the actual hydrogen ion concentration.

I therefore tried a buffer solution of formic acid and the easily soluble sodium salt of this acid.

Even the first trials succeeded surprisingly well and new investigations were started with formic acid of various normalities mixed with 1 N sodium formate in various proportions, in order to find a mixture with optimal decalcifying effect and maximal pH.

Of the various compositions, mixture No. 1 (equal parts of 1 N formic acid and 1 N sodium formate, pH 3.8) decalcified in 5 days, mixture No. 5 (equal parts of 2 N formic acid and 1 N sodium formate, pH 3.25) decalcified in 60 hours, mixture No. 8 (equal parts 4 N formic acid and 1 N sodium formate, pH 2.7) decalcified in 36 hours while mixture No. 10 (equal parts of 8 N formic acid and 1 N sodium formate, pH 2.2) decalcified in scarcely 24 hours.

In the investigation, the above mentioned bone blocks were used consisting of fresh *substantia compacta*, 2 mm. thick, with a diameter of 7.5 mm. and a weight of 170 mg. In all investigations the end of decalcification was controlled by means of X-ray photographs.

By simultaneous photometer examinations on preparations stained with Einarson's gallocyanin, lowered nuclear stainability was found in those which had been in mixtures with lower pH levels. However, the difference was not great; and therefore the fastest decalcifying mixture (No. 10) was preferred.

It is noticeable that the pH of this mixture of formic acid and sodium formate is the same as 1/10 N formic acid which, however, takes about 5 days for complete decalcification; and what is more strange, it decalcifies faster than 1 N formic acid with pH 1.75. Furthermore it decalcifies somewhat faster and much better than 1/10 N nitric acid or hydrochloric acid with pH 1.0 and, moreover, is apparently superior to the mixture of formic acid and sodium citrate mixture with pH 1.75.

Overdecalcification, by using twice the time necessary, shows a somewhat lowered nuclear stainability photometrically, but less so than other known decalcification agents under similar circumstances.

The treatment did not alter the color or the appearance of the bone blocks. They were found uniformly decalcified and easy to cut, even after embedding in paraffin. This is important for the problem under study, because embedding the labyrinths in paraffin is a necessary condition in carrying out the above mentioned staining method.

The only after-treatment used was washing in running water for 24 hours. The preceding treatment with sodium sulfate was omitted because a volumetric, pycnometric investigation showed no advantage from use of sodium sulfate, but rather sooner a distinct, though slight lowering of the stainability.

The method takes a somewhat longer time than the common 5% nitric acid procedure (double the time for guinea-pig labyrinths), but it allows greater possibility of employing special staining methods.

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LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

BOOK REVIEW

LILLIE, R. D. **Histopathologic Technic.** 6x9 inches. 300 pages. *The Blakiston Co., Philadelphia and Toronto.* 1948. \$4.75

This book was written to include recent advances in histopathological technic, especially to give definite and concise steps in procedures used either by pathologists or clinical technicians. It is intended to feature the shortest methods that give good results.

There are short concise chapters on microscopy, equipment, fixation and decalcification. Several other chapters treat enzymes, endogenous pigments and minerals, various cell products such as glycogen, chitin, cellulose and starch, amyloid and hyaline; and there is a lengthy chapter on fats and lipoids. One chapter takes up the subject of fibrin, bacteria, protozoa and other parasites; and another, glia, nerve cells and fibers. There is also included a section on hard tissue, and one on special procedures such as vascular injection, corrosion, autoradiography and microincineration.

A good share of the book deals with a wide range of the latest staining methods for special cells and tissue, including some new technics by the author, as well as modifications of well-known methods which he has modified in order to shorten the procedures without detriment to their efficiency. Throughout the book, the author has made valuable comments and suggestions on improving solutions and technics.

Among the most useful features are various tables. In the chapter on sectioning, for instance, there is a comparative table on dehydration, clearing and paraffin infiltration. There is also a schedule for diammonio silver hydroxide reticulum methods, comparing six methods, step by step; and another striking table is a comparative staining schedule for sequence with iron hematoxylin staining by five methods. The last chapter on buffers includes several valuable tables.—*M. A. Darrow.*

MICROSCOPE AND OTHER APPARATUS

BARRON, A. L. E. **Metal-coated mirrors.** *The Microscope, London*, 6, 225-6. 1947.

Three mirror coatings are discussed. Rhodium, which is electroplated onto a vacuum-deposited chemically inert film on a glass base, has a reflectivity over 70% and is very durable. "Chroluminum" consists of a triple deposit of chromium undercoating, aluminium reflecting layer, and protective silica finish and must be treated with reasonable care. "Duolux", a semi-transparent light-splitting film, can be cleaned with HNO_3 and detergents and is little affected by hard rubbing.—*C. Randall.*

FRISON, E. **A Leeuwenhoek microscope.** *The Microscope, London*, 6, 281-7. 1948.

The author gives a brief historical account of the sale of microscopes made by van Leeuwenhoek and information on the whereabouts of known surviving examples of the instruments. Six photomicrographs reproduced at 100 diameters from originals at 140 diameters taken by means of the original lens without the use of accessory apparatus are shown.—*C. Randall.*

MCARTHUR, JOHN. **Advances in the design of the inverted prismatic microscope.** *J. Roy. Micr. Soc.*, 65, 8-16. 1945.

The microscope in its over-all dimensions is 4x3x2 inches, made of fine metals, brass, manganese-bronze and stainless steel and is chromium plated throughout

which makes it resistant to various corrosive agents. All controls can be operated without moving the hand from the fine adjustment knob. There is no coarse adjustment. It is claimed it will do all that any conventional microscope will do and will do some things that the ordinary one is incapable of doing. The slide is inverted on the stage. The objective is below and the condenser is above. This necessitates a prismatic system (which lies in a prismatic light tube in the base of the scope) to direct the light from the inverted objective into a downward directed eyepiece. There is automatic focusing because the face of the slide always remains on the same plane as the stage. The "tube length" is 150 mm. Only a minimum of fine adjustment is necessary. There is no racking of the oil immersion objective away in order to change a slide. Images are erect. There exists a very simplified centering mechanism. There is a built-in illuminator, operated from a plug-in or from batteries, with a greatly improved control of illumination. A free interchange of condensers is possible. The focus of the condenser is fixed however. The microscope is admirably adapted for the hanging-drop technic. Microdissection, staining, and other manipulations are easily carried out with this miniature scope. The iris diaphragm is operated by the turning of the eyepiece. Objectives are changed by merely moving a vertical mount. One always knows the exact objective present by its feel. Binoculars may be inserted in the eyepiece tube. An attachment is being designed for photomicrography in such a way that the ordinary 35 mm. camera can be built in without interfering with routine work. This remarkable little microscope has had the test of the most rigid laboratory demands; it has stood up in all climates—on sea and land. It has even been run over by two wheels of an automobile without injury. There are five plates beautifully illustrating this instrument complete, and its separate parts. One full-scale drawing completes an excellent description.—*Paul G. Roofe*

SPENCE, D. S. Measurement of numerical aperture. *The Microscope*, London, 6, 253-9. 1947.

The author describes in detail the construction of an apparatus to measure the numerical apertures of "low power" objectives. A Beck objective, consisting of three lenses cemented together, and a Zeiss *a** objective are used. The microscope is set up with its tube horizontal and its mirror and sub-stage condenser removed. A thin card with a fine needle-hole is put on the stage and is brought into view through a "low power" objective and an eyepiece. The angle of the two extreme light rays is most easily found by measuring the angle through which the stand must be rotated to bring the image from one edge of the field to the other.—*C. Randall*.

TAYLOR, E. WILFRED. Improved image illumination and contrast with the metallurgical microscope. *J. Roy. Micr. Soc.*, 65, 1-7. 1945.

This article describes a well known practice of coating glass surfaces in order to reduce the reflected light and increase the transmitted light; this description is given primarily for the microscopist in his problems presented by the microscope objective dealing with the lighting of the object. A description is given of an experiment in which surfaces of four metals (three nonferrous, one ferrous) are used against stainless steel as a control. Both etched and unetched surfaces are photographed. It is pointed out that much can be done to improve the observing conditions when a glass cover illuminator is used. The metallurgist's requirements are given special consideration. There is a great reduction of fogging light due to the coating of glass surfaces, also a greatly improved contrast in the image and a more efficient operation of the cover glass illuminator.

Magnesium fluoride was applied to the internal surfaces, thus reducing surface reflections from 4 to 7% per surface to an average of about 1%. Other coating materials were used separately and in combinations giving varying results. Three plates with six figures give surface views of 12 different specimens with the percentage of harmful light present in each. Table I presents percentage of beneficial light reflected by the specimens before and after etching. Tables II, III, IV indicate the percentage of beneficial and harmful light passing through a Ramsden Circle with 16mm., 4mm., and 2mm., objectives respectively for both coated and uncoated surfaces of cast iron, phosphor bronze, cast brass and copper, the stainless steel acting as a control. By using a cover glass illuminator, it is

possible to increase the intensity of the image illumination as much as three times by the proper coating of the surfaces of the cover glass.—*Paul G. Roope.*

PHOTOMICROGRAPHY

NICHOLAS, J. W. The recording of exposure data in photo-micrography. *The Microscope, London, 6, 263-5. 1947.*

A system of recording exposure data in photomicrography which is adaptable to any method of illumination or microscopic observation is described. Type of apparatus, focal length of lenses, aperture, and focusing arrangements are recorded in sufficient detail that a new worker may exactly duplicate the conditions of any exposure with only the written record as a guide.—*C. Randall.*

MICROTECHNIC IN GENERAL

SPENCE, D. S. Heat-sealing fluid mounts. *The Microscope, London, 6, 227-9. 1947.*

The author describes a method for heat-sealing fluid mounts. The bottom of a cell is painted with shellac cement and allowed to dry for a week. It is then centered on a previously heated slip and firmly attached to it, cement side down. A coat of cement is applied to the top of the cell and around the edge of an appropriate cover. Both are allowed to dry.

The object is placed for one week in a watch glass in formalin 1 vol., glycerol 2 vol., and enough previously boiled water to make 40 vol. The cell is filled with glycerol 3 vol., water 1 vol., and the object inserted. The cover is then applied and the mount sealed by melting the shellac with a hot iron bar $\frac{3}{8}$ " in diameter tapered at one end.

The method is not recommended for sealing watery fluids such as dilute formalin.—*C. Randall.*

ANIMAL MICROTECHNIC

EARLE, WILTON R., SHELTON, EMMA, BROWN, MARY FRANCIS, and STRAUSS, NANCY. Procedure for the fixation, staining and mounting of whole mounts from tissue cultures grown in Carrel D 35 flasks. *J. Nat. Cancer Inst., 8, 83-9. 1947.*

The method describes adequately the procedure for making whole mount preparations of tissue cultures grown in Carrel D 35 flasks. This technic meets the need of proper fixation and preservation by which there is an insignificant distortion of tissue taken from tissue cultures. The fixative used is a formalin- K_2CrO_7 solution with a controlled pH. The fixative remains on the cultures about 15 min. in order to penetrate the plasma clot and kill the cells. The fixation is done at 38°C. and the rack is returned to the incubator. Several changes of the fixative are necessary. Great care must be taken in the removal of the tissues from the flasks. The material is hardened in 3% K_2CrO_7 for 5 to 8 days at 4°C. Washing in tap water for 24 hr. is suggested. Staining is done by a specially prepared iron hematoxylin designed for thick sections. Dehydration follows in the usual manner and clearing is done slowly through alcohols, acetone and toluene. Mounting is done in paper cells. Slides are seasoned for 60 days at 40°C. Cover slips are held down by lead weights.—*Paul G. Roope.*

HARTZ, P. H. Simultaneous histologic fixation and gross demonstration of calcification. *Amer. J. Clin. Path., 17, 750. 1947.*

The use of Bouin-Hollande solution is recommended for fixing tissues with simultaneous visualization of calcified structures. This solution is prepared as follows: Dissolve in the order named 2.5 g. copper acetate (probably cupric, but not specified) and 4 g. picric acid in 100 ml. distilled water without heat, filter, add 10 ml. of U.S.P. formaldehyde and 1.5 ml. of glacial acetic acid. This solution keeps indefinitely. Tissues are treated 8 hours to 3 days with this solution, then washed for several hours in 2-3 changes of distilled water. Calcified structures appear dark green on a pale green background. Tissues fixed in the above manner can be stained for other histological details with the trichome stain, for example.—*L. Farber.*

VERCOURT, B. The sectioning of beetle elytra. *The Microscope*, London, 6, 305-6. 1948.

A study of softening methods for hard chitinous insect tissues was made. Good softening after 5-6 days was obtained with a reagent composed of 24 ml. 50% HNO_3 and 1 g. KClO_3 . Schultze's reagent (2 ml. concentrated HNO_3 to 1g. KClO_3) produces softening after a few minutes, but is drastic and requires careful watching. Considerable softening can be produced with the following reagent: one vol. glacial acetic acid mixed with one part 20 vol. H_2O_2 . Following preliminary softening, elytra were washed, dehydrated in alcohol, and embedded by the celloidin method.—C. Randall.

WILLIS, A. G. A new method for staining neurofibrillae and axis-cylinders. *J. Roy. Micr. Soc.*, 65, 29-33. 1945.

In the introduction the author states that this is a rapid and certain method for demonstrating neurofibrillae and axis-cylinders in vertebrate material. Considerable discussion is presented for this "rational histological process for the demonstration of basic proteins with the general characters of histone or protamine". It is stated that these basic proteins have been recently discovered in the axoplasm extruded from the giant nerve fibers of the cephalopod mollusc, *Loligo*. The author's rationale includes the probable reaction of the fixative (formalin) with protein, resulting in the formation of additive compounds with the amino groups. The following is an outline of the method recommended: (1) Preliminary treatment: the fixative consists of "20% commercial (unneutralized) formal" (probably 8% formaldehyde) to which has been added 3 ml. of ammonia water Sp. Gr. 0.880 in distilled water (probably to 100 ml. of the formalin); 30 ml. of this mixture is added to 70 ml. of absolute alcohol. The minimum period of fixation is 18 hr. (2) Secondary treatment with formalin and then acid or alkali treatment as follows: Bring sections down to water. Place 1 hr. in "20% formal" in distilled water to which 6 small drops of ammonia water Sp. Gr. 0.880 are added (per 100 ml.?). Give a second treatment with this same solution diluted 1:50. Rinse $\frac{1}{2}$ to 1 min. in distilled water, then transfer to one of the following acid or alkali solutions: (a) Acetic alcohol—25 ml. of glacial acetic acid to 75 ml. absolute alcohol; (b) 2 ml. glacial acetic acid to 90 ml. of 90% alcohol; (c) Ammoniacal alcohol—2 ml. of ammonia water Sp. Gr. 0.880 to 98 ml. of 90% alcohol; (d) Solutions of sodium, magnesium or lithium carbonates; add a 1% stock solution of one of these in distilled water drop by drop to 100 ml. distilled water until the pH reaches 8-10. In taking sections through these solutions the usual methods of upgrading or downgrading are carried out. (3) Silvering and primary reduction: pass sections for 5-20 min. into 1% solution of AgNO_3 in the dark at 38° C.; wash 1-3 minutes; reduce 1-5 min. in a solution of hydroquinone, 1 g., sodium sulfite (cryst.), 10 g., formalin, 4 drops, distilled water, 100 ml.; wash 5 minutes. (4) Toning and final reduction: pass for 10 min. into gold chloride (1:600 solution) 100 ml., glacial acetic acid, 4 drops; wash in distilled water; pass into 2% aqueous oxalic acid for 15 min. for final reduction; wash as usual. (5) Mount either with or without counterstain. Treat celloidinized sections similarly.—Paul G. Roope.

PLANT MICROTECHNIC

BOND, T. E. T. The "phloem necrosis" virus disease of tea in Ceylon. *Ann. Appl. Biol.*, 34, 517-25. 1947.

To identify phloem necrosis in tea, whole leaves or leaf-tips were placed in 50% alcohol; freehand sections were cut, stained rapidly in light green (FSY?) in clove oil, and mounted in Canada balsam. The sections were stained just sufficiently to show the difference between necrotic and other cell walls. In "true necrosis" caused by a virus the cells of the protophloem are killed and obliterated; this destruction then extends outwards to the pericycle and inwards to the metaphloem; the destruction of these areas is followed by the production of new thin-walled cells by hyperplasia. Non-pathogenic "false necrosis" originates in the metaphloem and shows no hyperplasia. The two types can readily be distinguished in the petiole but not in the midrib.—H. P. Riley.

MICROÖRGANISMS

MAY, J. **Einfache Sporenkontrastfärbung mit Oxyppyrentrisulfosäure.** *Zentbl. Bakt., I Abt. Orig.*, 152, 56-7. 1947.

A procedure is described to achieve a good spore stain which also can be used to demonstrate the polar bodies of the diphtheria organism in the fluorescent microscope. The sodium salt of 3-oxypyrene-5, 8, 10-sulfonate, is used. The alkaline dilution of this dye gives a green, the acid solution a blue luminescence of bacteria with intensive prominence of spores and bipolar bodies in ultra-violet light. By the staining of smears with a mixture of acid oxypyrene and acridine orange, a striking contrast stain is achieved. The bacteria shine red brown, the spores blue in ultra-violet light.—*Ivan Saphra*. (Courtesy *Biological Abstracts*).

YEGIAN, D., and VANDERLINDE, R. J. **The nature of acid-fastness.** *J. Bact.*, 54, 777-83. 1947.

The dyes in the Ziehl-Neelsen technic are believed to accumulate within the cell wall and to be held there in an unbound form which can be precipitated as granules under certain conditions. These granules can be redissolved inside the cell to permit it to present a uniformly stained picture again; or the excess dye can be removed leaving the cells faintly pink, a color attributed to the bound dyes. The observations were made on both acid-fast bacteria and fungus spores.—*Virgene Kavanagh*.

YOUNG, G. **Pigment production and antibiotic activity in cultures of *Pseudomonas aeruginosa*.** *J. Bact.*, 54, 109-17. 1947.

The pigment pyocyanin inhibits *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium smegmatis*. Pyocyanin breaks down into α -xyphenazine in chloroform but not in water.—*V. Kavanagh*.

HISTOCHEMISTRY

EWER, D. W., and HANSON, JEAN. **Some staining reactions of invertebrate mucoproteins.** *J. Roy. Micr. Soc.*, 65, 40-8. 1945.

In a carefully prepared table, the author lists the staining reactions of mucus glands and annelid tubes in 18 different species of invertebrates covering four phyla; two mammalian species and one amphibian also are listed. The stains are mucicarmine, thionin, Weigert, orcein and safranin. A description of each tissue from each species is also presented. The author, using Hempelmann's toluidine technic, showed that the reactions given by the polychaete glands were identical with those of the oral glands of the mouse which shows that they contained mucoitin, and not chondroitin-sulfuric acid. They further pointed out that the mucoproteins of invertebrate tissues, notably from polychaeta worms, are stained metachromatically purple with Weigert's resorcin-fuchsin and metachromatically orange with safranin and are stained by orcein. These properties are not possessed by the mucoproteins of most of the other animals examined by the authors.

It is pointed out that the property of all mucoproteins to stain metachromatically with suitable basic aniline dyes accounts for the resorcin-fuchsin and safranin reactions. There are a diversity of mucoproteins in the animal kingdom whose nature awaits investigation through these staining reactions.—*Paul G. Roope*.

STAIN TECHNOLOGY

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BASIC FUCHSIN AS A NUCLEAR STAIN

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ABSTRACT.—Five distinct nuclear stains and staining procedures which utilize basic fuchsin as the dye have been studied, compared and tested on a Feulgen-weak fungus, *Blastomyces dermatitidis*, and other fungi.

Aqueous basic fuchsin has been shown to be an excellent, though impermanent, stain with which to study the nuclei of this and other fungi. The conditions under which formaldehyde acts as a mordant for basic fuchsin and produces a permanent nuclear stain have been established.

Comparison of crystal violet and basic fuchsin suggests that the mordanting action of the aldehyde operates through the para-amino groups of the dye. Certain other basic dyes were not mordanted by formaldehyde.

Gentle acid hydrolysis of the tissues has been found to be essential both to the specificity of the dye as a nuclear stain and to the mordanting effect of the aldehyde.

The possible relationship of these observations to the Feulgen reaction is discussed. A protocol for the method developed is presented.

In this report certain conditions under which basic fuchsin becomes a useful and versatile nuclear stain, especially for Feulgen-weak and Feulgen-negative organisms, will be described. Studies of this subject were undertaken as a consequence of investigation of certain fungi pathogenic for man and animals which have been found to give poor or negative reactions with the Feulgen technic, when routine procedures were used, and for which this technic is not usable in the usual cytologic studies. For these same organisms even the best of the iron alum hematoxylin preparations frequently have a muddy or cloudy aspect not good for detailed cytologic work. It is believed that at least a partial solution of the difficulties has been

found. The results of the studies, however, present difficulties of interpretation, especially as related to the Feulgen reaction, which are not met easily.

It is not our intention to enter into the current discussion concerning the specificity of the Feulgen reaction as a nuclear stain nor to summarize the literature. It is believed, however, that the observations reported herein will be of interest to those active in this work. The results of our studies suggest that work on organisms, giving a weak or negative Feulgen reaction, or on the Feulgen-negative phases of the nuclear cycles of certain organisms may lead to further elucidation of some of the problems relative to the specificity of the Feulgen and other nuclear stains.

HISTORICAL DATA

Empirically it would appear that material fixed in formalin when stained with certain dyes gives more brilliant results than the same material fixed with some other fixing agent. So far as we can determine from the literature, this suspicion has not been analyzed or elaborated except in a single instance. Ohlmacher (1895) observed that formalin could be used as a mordant in the Ziehl carbolfuchsin stain for tubercle bacilli and that it prevented decolorization by the usual methods. He also observed that the nuclei of the pus cells in smears so treated retained an intense and brilliant stain. He applied a 2 to 4% solution of formalin as a mordant to material to be stained with aniline-water-gentian-violet, aqueous methyl violet or aqueous methylene blue, and also used a 4% solution of formalin as a base for his staining solutions. He found that these methods and mixtures gave intense and powerful stains for bacteria as well as for animal tissues.

Since these initial observations, formalin appears to have been used primarily only in its capacity as a fixative and preservative or as a test agent in recent studies on the specificity of the Feulgen reaction (Choudhuri, 1943; Stowell, 1945, 1946).

Stowell (1945, 1946), among others (Bracket, 1944, 1946; Caspersen, 1944; Choudhuri, 1943; Dodson, 1946; Stacey, Deriaz, Teece and Wiggins, 1946; Stedman and Stedman, 1943, 1944), recently has reviewed and commented on the pertinent observations and opinions concerning the specificity of the Schiff (Feulgen) reagent and the nature of its reaction as applied to tissues (Barger, and Callan, 1944; Callan, 1943; Serra, 1943). The sum total of these observations tend to confirm and extend the original observations of Feulgen (1932) and his various co-workers (1924). Only those of such observations pertinent to the present subject will be more specifically mentioned.

Basic fuchsin has long been known as an excellent nuclear stain (Lee, 1937) and it is used in its chromatic form in numerous stains and bacteriologic media. In its leuco form, produced by the action of sulfurous acid, this dye forms the Schiff reagent, which when applied to tissues becomes known as the Feulgen stain (Wieland and Scheuing, 1921; Feulgen and Rossenbeck, 1924). Stowell and others (1943, 1945, 1946), using spectrophotometric methods, demonstrated differences in the absorption curves of basic fuchsin in its chromatic and its leuco forms. They showed that the Schiff reagent, in which the addition of aldehyde (which they considered to be an addition compound) has induced color, has an absorption curve distinct from that of basic fuchsin in either its chromatic or its leuco form. Stowell and Albers (1943) further demonstrated that this compound has an absorption curve similar to that of thymus gland stained with the Feulgen stain. Stowell (1945, 1946) and Baker (1942) considered a positive Schiff or Feulgen reaction to be due to the formation of an addition compound between the aldehyde and the leuco form of the basic fuchsin.

Baker (1942), Conn (1946) and Karrer (1946) stated that the change in color from the chromatic form to the leuco form of basic fuchsin, induced by sulfurous acid, is due to the loss of the quinonoid linkage, and that regeneration of color is due to the reformation of this linkage as shown in the accompanying structural formulas (Fig. 1, 1 and 2). Related structures are shown as 3, 4 and 5 of Figure 1.

The Feulgen reaction requires acid hydrolysis of the tissue in order that the staining reaction may occur. This hydrolysis purportedly frees an aldehyde radical in the desoxyribonucleic acid. The Feulgen reagent supposedly forms a chemical union with this aldehyde radical in the manner shown in Figure 1, 5. According to Baker (1942) and Karrer (1946) evidence has been offered that such a chemical reaction occurs.

On the other hand it must be noted that the effects of gentle acid hydrolysis on the complex organic substances (including desoxyribonucleic acid) which comprise the nucleus, are not yet clearly known and that specific applicable information is scant. Absolute assurance therefore that the mechanism just described operates in the Feulgen reaction is lacking.

Although Stowell and others (1943, 1945, 1946) have demonstrated spectrophotometric differences in the absorption spectra of the Feulgen reagent, basic fuchsin and the compound formed by the addition of aldehyde to the Feulgen reagent, Choudhuri (1943) considered the staining effect of the last to be identical to that of the Feulgen reagent. Carr (1945) suggested a similar equivalence with

basic fuchsin acidified with hydrochloric acid for tissues hydrolyzed by acid. He apparently considered this to be a valid Schiff reagent. Neither Carr (1945) nor Choudhuri (1943) considered the Feulgen stain to be specific. Since Ohlmacher (1895) no one appears to have used formaldehyde as a mordant prior to staining with basic fuchsin. Choudhuri's colorized Schiff reagent, however, is similar to Ohlmacher's formalinized basic fuchsin solution, but is not identical with it.

It seems likely that the differences of opinion expressed by the various groups working on this subject rest not on incorrect and unrelated observations, but on differences of interpretation of observed phenomena, as well as on lack of certain crucial information concerning especially the specific effects of acid hydrolysis on the nuclear complex and the specific chemical reactions occurring within the Schiff reaction.

MATERIALS AND METHODS

Basic fuchsin has been studied in our laboratory in what appears to be five distinct chemical states. These five chemical forms are basic fuchsin (the various rosanilines), the Feulgen or Schiff reagent, the Feulgen reagent plus aldehyde, basic fuchsin plus hydrochloric acid (constituting a Schiff reagent?) and basic fuchsin plus aldehyde. The results of staining with each of these chemical forms of the dye need not be comparable and indeed are not, as will be seen presently. This will become more apparent as the data are presented.

It should be mentioned in passing that the nuclei of many organisms are known to stain very weakly or not at all with the Feulgen stain. This subject will form the basis for a separate report. Certain organisms, however, have been found to give positive staining reactions, after proper conditions for operation of the stain have been discovered (DeLamater; Rafalko, 1946). It likewise seems quite possible that the cytologist in seeking a usable technic has disregarded weak positive reactions and considered them negative because they did not satisfy his particular need (Boas and Biechele, 1932).

Material studied.—The following organisms have been used as test objects: *Blastomyces dermatitidis*, fourteen strains from various sources, and a single strain of *Candida albicans*, *Candida albicans*, variety *stellatoidea*, *Candida albicans*, variety *Candida*, *Candida Krusei*, *Candida parakrusei*, *Histoplasma capsulatum* and *Saccharomyces cerevisiae* and many others to be reported on at a later date. Tissue sections have been obtained from a dog with blastomycosis and smears and tissue from a mouse that had experimental sporotrichosis. All of these organisms were found to give positive staining reactions under the conditions to be enumerated. Blastomyces has been used most extensively, however.

Several of the fungi grow in two phases, the yeastlike phase and the filamentous phase. Each phase can be induced by growing the organisms on the proper medium and at the proper temperature. The yeastlike or budding phase was grown in test tubes, fixed *in situ* with Schaudinn's fluid (see "Fixation"), immediately stirred into suspension with a platinum loop and poured into 15 ml. centrifuge tubes. All procedures were carried out in these tubes. Solutions were changed by rapid centrifugation and decanting of the supernatant fluid after which a fresh solution was immediately added. Undoubtedly some of the findings reported herein would not have been made

had this test tube method not been used. These procedures are slower and necessitate longer exposure of the cells to reagents and solvents than some other procedures.

In the filamentous phase, *Blastomyces* and *Histoplasma* were grown on cellophane sheets placed over a medium which permitted adequate, but not too thick, growth for observation through the mycelial mat. These cultures also were fixed *in situ* with Schaudinn's solution and the mycelium was transferred to a Petri dish. All procedures were applied by changing the solution. At the time of mounting, the mycelium was peeled from the membrane and mounted right side up in Clarite or balsam. Permount was found to be an unsatisfactory mounting medium, producing severe shrinkage of the protoplasts. Tissue sections and smears were handled in the classic manner in Coplin jars.

All tissues were dehydrated through alcohol in 10% stepwise dilutions and cleared through xylene.

Fixation.—Schaudinn's solution, Feulgen's fixative and Zenker's fixative were tested. Schaudinn's solution gave the least shrinkage and best nuclear detail and, therefore, was used exclusively in subsequent studies.

Stains.—Several lots of basic fuchsin were tried, all successfully. Basic fuchsin supplied by the National Aniline Division of the Allied Chemical and Dye Corporation with a certification number of NF-54 has been used most extensively.

The following dyes were studied for purposes of comparison: methyl violet 6B. of Dr. G. Grüber and Co.; azure I (Giemsa) of Dr. G. Grüber and Co.; methylene blue chloride with a certification number of NA-26 of the National Aniline Division of the Allied Chemical and Dye Corporation; Bismarck brown Y of Coleman and Bell, and Lichtgrün of Hollborn and Sohne, listed as an "I.G.F. standardized stain."

The last-named dye was used for counterstaining when this procedure was tried or deemed advisable.

All stains were used in 0.5% solutions made up in distilled water unless otherwise specified. Distilled water was used in the preparation of all solutions.

The pH determinations were done with a Beckman pH meter model G. The inaccuracies of this method in the lower pH ranges is acknowledged.

Destaining.—Destaining was accomplished by soaking the stained cells in distilled water and in the alcohol-water dilutions, controlling the degree of destaining by the length of exposure to the water or alcohol-water dilutions as indicated by direct observation of the cells through the microscope. Acid-alcohol and Feulgen bleaching solutions also were found to be useful as destaining agents, but their action could not be controlled sufficiently well on cells handled in test tubes to warrant their use under these conditions.

Experimental data.—The studies carried out on the various basic fuchsin stains will be described under separate headings. In the instances in which a useful protocol was worked out it will be presented in the appropriate section.

THE FEULGEN REACTION

The Feulgen stain was used according to the routine procedure which consisted of hydrolysis in normal hydrochloric acid for five minutes at room temperature, for five minutes at 60°C. and then for five minutes at room temperature; washing of the cells in distilled water, and application of 0.5% basic fuchsin decolorized with 0.5 g. of sodium sulfite plus 10 ml. of normal hydrochloric acid. Rafalko's method of decolorization also was used with success, as would be ex-

pected chemically, since sulfur dioxide is the desired active chemical, whatever its source.

Modifications of the procedure consisting of changes in the concentration of the stain and sulfite did not affect the staining reaction or decolorization, so long as the stain was not in excess. With the stain in excess intense but impermanent bright red staining of the nuclei occurred; this color readily washed out in alcohols. Such staining was found to occur with plain 0.5% aqueous solution of basic fuchsin.

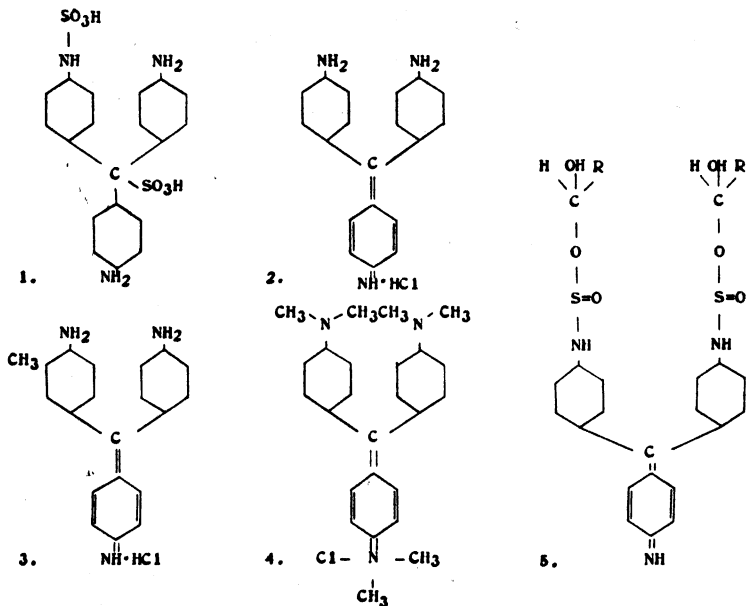


FIG. 1.—1. Schiff's or Feulgen's reagent. Colorless form showing loss of quinonoid linkage with the formation of a sulfurous acid salt. 2. Pararosaniline (magenta O), chromatic form showing quinonoid linkage. 3. Rosaniline (magenta I), chromatic form showing difference from pararosaniline in the presence of a methyl group attached to one of the phenyl rings. The rosanilines (magentas) differ one from another in the number of such methyl groups. 4. Methyl violet, a dye of the same chemical series as the magentas in which the para-amino groups are blocked by methyl groups. No mordanting effect of formalin occurs when these para-amino groups are thus blocked. 5. Postulated configuration of linkage between Feulgen's reagent and the desoxyribonucleic or thymonucleic acid. The R represents the desoxyribonucleic or thymonucleic acid component. The quinonoid linkage is reformed.

It was incidentally noted that overhydrolysis eliminated both the Feulgen reaction and staining with iron alum hematoxylin, in confirmation of Stowell (1946).

Because of extensive studies on the Feulgen reaction since the initial writing of this paper, the detailed material on this subject will be submitted in a subsequent report. It would appear that at least a partial explanation for aberrations in the Feulgen reaction has been obtained.

BASIC FUCHSIN STAIN

Aqueous solution of basic fuchsin.—Several interesting and useful observations were made at the time the experiments on the Feulgen reaction were being conducted. The nuclear structures in material hydrolyzed as for the Feulgen stain and stained with 0.5% aqueous solution of basic fuchsin or with the Feulgen reagent which had been allowed to colorize by loss of sulfur dioxide stain an intense bright red. Cells which had not been hydrolyzed stained poorly and nonspecifically. It was found that acid hydrolysis (standard procedure) eliminated much of the nonspecific staining of cytoplasm with aqueous solution of basic fuchsin and apparently produced a specific effect on the nucleus which intensified the nuclear stain. It was found, however, that the stain produced by aqueous solution of basic fuchsin faded slowly in aqueous solution and extremely rapidly in alcoholic solutions, so that dehydration through the alcohols could not be accomplished. Feulgen's sulfur dioxide bleach solution or acid alcohol removed this basic fuchsin stain almost instantaneously.

It was found, however, that hydrolyzed material, stained in aqueous solution of basic fuchsin, could be run through solutions of sugar and gum arabic of varying strengths, and temporary mounts thus could be made which would last four to six weeks. If during the mounting procedures too great destaining occurred, the nuclei could be restained by adding dye to the menstruum of sugar, water and gum arabic.

This procedure offers great promise as an aid in the study of nuclear phenomena in fungi. Direct and immediate observation of the cells in water without the mixture of gum arabic and sugar also can be made.

The formulas for the mounting media were taken from Lee (1937) and modified to meet the requirements of these studies.

Attempts to stain the test fungi with basic fuchsin in alcoholic solution produced intense staining of the walls of the cells with no visible differentiation of cytoplasm and nuclei. The same was true if the cells were fixed, dehydrated in 70% alcohol and then hydrolyzed in 70% alcoholic, normal hydrochloric acid.

Effect of preliminary hydrolysis and mordanting of cells in formalin and of acidifying the staining solution.—When numerous efforts to obtain satisfactory cytologic preparations of *Blastomyces* and other fungi with the Feulgen stain failed, the observed staining of nuclei by simple aqueous basic fuchsin was studied in detail. After it was learned that fading of such preparations occurred, a mordant was sought.

The permanganate method of mordanting (1% permanganate for one minute) was tested. From this concentration marked precipi-

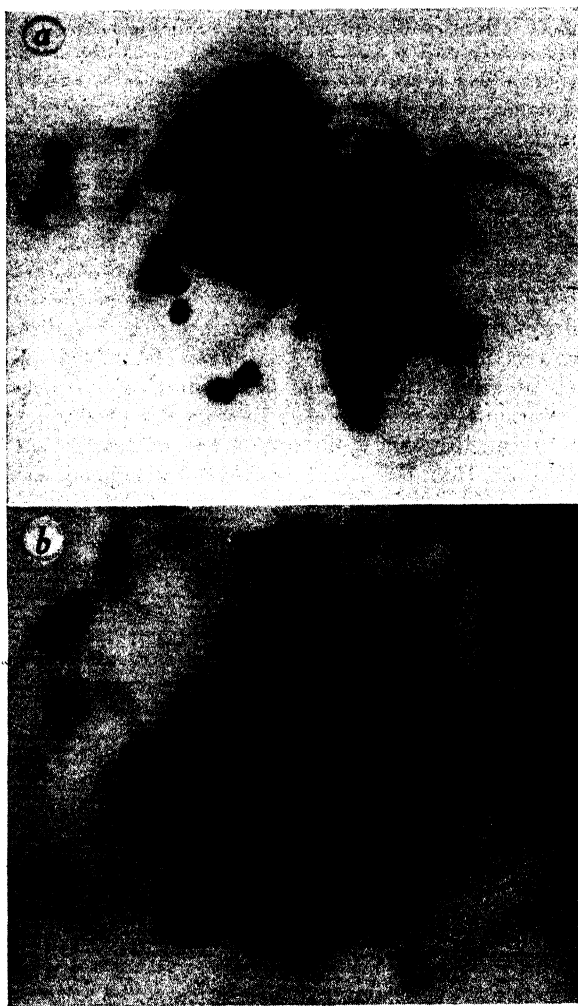


FIG. 2.—*a*. Resting nuclei in budding phase of *Blastomyces dermatitidis* (basic fuchsin, formalin mordant $\times 3,400$). *b*. Tuberculate chlamydospore of *Histoplasma capsulatum* showing early resting nuclei (basic fuchsin, formalin mordant $\times 3,400$).

tation occurred about the cells; the walls were markedly discolored (brown) and there was severe shrinkage of the protoplasts. No

staining occurred after this procedure. All concentrations of potassium permanganate from 0.0625 to 4% were found to be equally ineffective and destructive.

Ohlmacher (1895) described both the incorporation of aldehyde into the staining solution and the process of mordanting cells in 2 to 4% solution of formalin prior to staining. Certain stains and the mordanting effect produced by formaldehyde with each will be presented in addition to the studies which define the use of basic fuchsin.

It was found that gentle acid hydrolysis was necessary to a clear and precise nuclear stain with aqueous basic fuchsin (see above). To ascertain whether acid hydrolysis was also necessary to the mordanting effect of formaldehyde, as well as to the staining, experiments were set up as follows:

1. Cells were fixed in Schaudinn's solution, but were neither hydrolyzed in normal hydrochloric acid nor mordanted in aldehyde.
2. Cells, after fixation, were both hydrolyzed and mordanted. Hydrolysis consisted of exposure to normal hydrochloric acid for five minutes at 30°C., for five minutes at 58°C. and for five minutes at 30°C. Cells were rinsed in distilled water prior to mordanting. Mordanting consisted of exposure of hydrolyzed cells to 2% solution of formalin for two minutes, followed by washing in distilled water.
3. Cells, after fixation, were hydrolyzed, but not mordanted.
4. Cells were not hydrolyzed, but were mordanted.

All cells then were stained for fifteen minutes in 0.5% aqueous solution of basic fuchsin, washed in distilled water and passed through the alcohols. Destaining was accomplished simultaneously with dehydration. The length of exposure in any dilution of alcohol and water was determined by the degree of destaining.

Cells which were not hydrolyzed or mordanted stained diffusely. There was no selective staining of the nuclei. Furthermore, what staining occurred, rapidly faded on passage through the alcohols.

Hydrolyzed and mordanted cells stained intensely and the nuclei stained much more intensely than the cytoplasm. In the alcohols the cytoplasmic stain was removed and clearly, precisely and persistently stained nuclei remained (Fig. 2*a* and *b*). In cells that were hydrolyzed, but not mordanted, the nuclei stained clearly and selectively but faded rapidly in the alcohols (see section on "Aqueous solution of basic fuchsin"), while cells that were not hydrolyzed but were mordanted stained intensely but unselectively, there being heavily stained masses and amorphous granules in the cells. The stain in these cells faded rapidly in the alcohols and shrinkage of protoplasts was severe.

The results with animal tissues differ somewhat from those obtained with the fungi. A clear-cut nuclear stain is obtained with aqueous basic fuchsin. The cytoplasm does not appear to take up the stain so avidly either in the absence of hydrolysis or when aldehyde mordanting alone is done. The combination of hydrolysis and aldehyde mordanting necessary to the reaction with fungi apparently produces, with animal tissues, no effect greater than either procedure alone. Whether or how the linkage of the dye with the nucleoprotein (or nuclear components) differs in these three circumstances is not clear. Nor is it clear in what manner the fungous cell differs so markedly from mammalian tissues. It is hoped that further studies may clarify the problem.

It also must be pointed out that addition of aldehyde to cells already exposed to aqueous solution of basic fuchsin did not produce a mordanting effect. The mordanting effect appeared to be blocked. However, if the stain is removed sufficiently by washing, aldehyde mordanting is then possible. Conversely the mordanting aldehyde can be sufficiently washed out so that no mordanting effect is obtained when dye is added.

Exposure of cells, mordanted with aldehyde and stained with basic fuchsin, to aldehyde did not cause destaining as exposure of material stained with hematoxylin to iron alum does.

In order to ascertain whether washing of hydrolyzed cells in distilled water prior to mordanting and staining changed their staining proclivities, experiments were set up in which cells were mordanted directly without washing and mordanted after one, two and three washings. The pH of all solutions removed from the cells was determined by means of a Beckman pH meter. Although a mild precipitate formed in the unwashed cells, nuclear staining was clear and precise. In all other series no precipitate developed and nuclear staining was excellent. It appeared, however, that the less acid the cells became, the redder the resultant stain and the less persistent in alcoholic solutions.

It was found that if the solution of basic fuchsin was made slightly acid, with a normality of 0.04 or 0.02, the stain was consistently more intense, was magenta and, if hydrolysis and mordanting preceded staining, was resistant to decolorization.

To ascertain the optimal concentration and duration of exposure of formalin as a mordant, 1, 2, 4, and 6% solutions were tested for varying periods. Although fairly comparable results were obtained with the 1, 2 and 4% solutions acting for periods of two to eight minutes, the best results were obtained consistently with a 2% solution acting for four minutes. Large concentrations and longer

periods of action tended to produce a precipitation about the cells when the stain was applied.

Comparison of basic fuchsin with other stains.—Five basic stains representing three chemical classes of dyes were chosen. These were Giemsa's azure I, methylene blue, Bismarck brown, crystal or methyl violet, and basic fuchsin.

Azure I and methylene blue are members of the quinone-imine group. Bismarck brown is included by Conn (1946) among the azo dyes; basic fuchsin and crystal violet belong to the triphenylmethane dyes.

Azure I according to Conn (1946) contains a predominance of azure B which is a trimethyl derivative of thionin, while methylene blue differs from this dye only in the presence of an additional methyl group. Basic fuchsin is considered to be a mixture of pararosaniline (magenta O), rosaniline (magenta I) and magenta II. These three dyes differ from one another only in the presence of methyl groups attached to the phenyl rings. They all have two amino groups in the para position (Fig. 1, 2 and 3). Crystal violet differs from the magenta dyes in that the hydrogen atoms in the two para-amino groups in this dye are replaced by methyl groups (Fig. 1, 4).

Cells treated with (1) azure I or methylene blue or (2) crystal violet were manipulated prior to staining as follows: (a) hydrolyzed according to the routine procedure and mordanted in 2% solution of formalin for two minutes; (b) hydrolyzed but not mordanted; (c) not hydrolyzed but mordanted, and (d) not hydrolyzed and not mordanted.

Beautiful and precise nuclear staining occurred with both groups of dyes with procedures a and b while in aqueous solution. Mordanting did not appear to affect the results. However, cells treated by both procedures a and b faded rapidly in alcohol-water mixtures, and it was obvious that mordanting did not affect the resistance of the dyes to the alcohols. Cells which were not hydrolyzed but were mordanted stained diffusely and nonspecifically and also faded rapidly on washing in the alcohols. In untreated cells exposed to azure I or methylene blue, only cytoplasmic granules stained and these also faded in the alcohols. (See section on "Mixture of basic fuchsin and aldehyde.")

Cells exposed to Bismarck brown, no matter how treated, did not stain, although the walls were discolored. Cells exposed to basic fuchsin reacted in the manner already described in detail. We, therefore, are unable to confirm Ohlmacher's contention that crystal violet or the azure dyes, at least under the conditions of these experiments, are mordanted with formaldehyde. Mordanting with formaldehyde, however, was not tried with aniline water and crystal violet which Ohlmacher used. A further consideration of the significance of these findings is presented in the comment.

Protocol for basic fuchsin stain following acid hydrolysis and formalin mordanting:

1. Fix cells in Schaudinn's solution.
2. Hydrolyze cells for five minutes at room temperature, five minutes at 60°C. and five minutes at room temperature.
3. Wash cells one to three times in distilled water.
4. Mordant cells in 2% solution of formalin for four minutes [2% formalin is made by diluting formalin (10% formaldehyde) to 2% with distilled water].
5. Wash cells in distilled water.
6. Place cells in 0.5% basic fuchsin in 0.04 normal hydrochloric acid for fifteen minutes. (This solution can be made up easily and approximately without too gross dilution of stain by adding 0.2 ml. of normal hydrochloric acid per 5 ml. of 0.5% aqueous solution of basic fuchsin. This works as well as when dye is made up to proper concentration in 0.04 normal hydrochloric acid.) Lower concentrations of dye can also be used to advantage.
7. Wash in distilled water.
8. Dehydrate and simultaneously destain in alcohols.
9. Clear in xylene.
10. Mount in Clarite or balsam.

Mixture of basic fuchsin and aldehyde.—Basic fuchsin with formalin (2 to 4%) incorporated in the stain was used by Ohlmacher. Ohlmacher also used methylene blue and crystal violet in a similar manner, but added aniline water.

When formalin is added to an aqueous solution of basic fuchsin, a change of color occurs from the usual red of basic fuchsin to a magenta color. No reference to this color change has been found in the literature. Such a stain, in which the concentration of basic fuchsin is 0.4% and of formalin is 2%, stains the nuclei clearly and selectively and the resultant stain resists decolorization when exposed to mixtures of alcohol and water, and decolorizes slowly in acid alcohol. Precipitation of stain, however, was encountered when the stain was applied to cells retaining too much acid from the hydrolysis procedure, despite the fact that the cells had been washed in distilled water. It was found that if the cells were washed sufficiently (three times) so that their pH was increased to more than 3.0, and if the basic fuchsin was made up in 0.01 normal solution of hydrochloric acid containing 2% formalin with a pH of 3.4, less precipitation occurred. If, however, the pH was allowed to remain very low (pH of about 1) or if phosphate buffers were added to stabilize the pH at a much higher range (pH 4.7 to 5.2) marked precipitation resulted and no staining occurred.

Because of the difficulties of controlling this precipitation of stain it was found easier to mordant the cells in 2% formalin and then expose them to a solution of basic fuchsin of known pH. This procedure and the experiments on which it is based have been described.

A comparison was made of the staining reactions of the basic-fuchsin-aldehyde mixture and the aldehyde colorized Schiff reagent (Schiff reagent with aldehyde added). Both solutions contained the same concentrations of dye, 0.4%, and formalin, 2%. They differed only in that the Schiff reagent had been previously decolorized with sulfur dioxide for twelve hours before the aldehyde was added. Whereas the basic-fuchsin-aldehyde mixture (with pH at approximately 3.5) gave a highly selective and resistant stain, the Feulgen-aldehyde mixture stained in a more generalized manner affecting the cytoplasm as well as the nuclei. It faded rapidly in alcohol-water mixtures and no permanent stain was obtained.

A comparison of the basic-fuchsin-aldehyde mixture with similarly treated solutions of Giemsa's azure I, methylene blue and methyl violet, gave the following results.

Basic fuchsin was the only one of these stains which changed color on the addition of aldehydes. Clear-out, precise and specific nuclear staining occurred with methyl violet and basic fuchsin only. No staining occurred with the methylene blue dyes. Staining of nuclear structures did, however, occur with all dyes, including the methylene blues, when the dyes were applied in simple 0.5% aqueous solution and the aldehyde was applied as a mordant prior to staining.

The aldehyde, whether it was included in the stain or applied to the cells previously, caused resistance to decolorization only when basic fuchsin was used. In other words all stains except basic fuchsin faded rapidly when exposed to alcohol and water in various dilutions, to acid alcohol or to Feulgen's bleach solution.

It is interesting that (as with basic fuchsin) if no acid hydrolysis preceded the staining—whether the cells were treated with aldehyde or the aldehyde was included in the stain—only a diffuse, non-specific staining of the cells and cell walls occurred.

COUNTERSTAINING

Counterstaining with fast green FCF was tried. It was found that the fast green tended to cover or replace the basic fuchsin in preparations of fungous cells and that the delicate nuclear structures were obscured. The cell walls, however, were stained intensely with the green dye and the cells were delineated clearly by this means. In tissue sections counterstaining with fast green produced a delicate and useful contrast.

COMMENT

What appear to be five distinct stains and staining procedures in which basic fuchsin is the dye used have been studied as applied to both fungi which give Feulgen-weak reactions and to tissues. These stains are the Feulgen stain, the Schiff reagent colorized with aldehyde, aqueous basic fuchsin, basic fuchsin decolorized with hydrochloric acid, aqueous basic fuchsin with aldehyde added in a dilute acid menstruum (0.04 normal hydrochloric acid) and aqueous basic fuchsin in dilute acid menstruum preceded by a formaldehyde mordant. It seems likely that the last two stains are in reality modifications of the same chemical process. That the Feulgen reagent and basic fuchsin decolorized with hydrochloric acid can be considered as distinct compounds seems probable. In the experiences reported herein parallel results were obtained with both. With neither was adequate nuclear staining obtained under the conditions of these experiments with Feulgen-weak organisms. In what manner Feulgen reagent colorized with aldehyde and aqueous basic fuchsin to which aldehyde is added differ from one another chemically is not clear. Both substances change color (become magenta) in the presence of aldehyde. That they differ is indicated by the disparity between the staining reactions obtained with each. No photometric analysis of possible differences between them was done.

Aqueous basic fuchsin (in 0.04 normal hydrochloric acid) preceded by use of formaldehyde as a mordant and basic fuchsin (in 0.04 normal hydrochloric acid) to which the aldehyde is added have been studied in detail and the conditions under which they can be used have been elaborated. Certain points deserve emphasis. For each of these stains, as well as for the Feulgen reaction, acid hydrolysis of the cells is necessary to the specificity of the staining reaction. For permanence of the stain, formalin must be used as a mordant, either applied to the cells before the stain, or incorporated in the stain. Acid hydrolysis is as important to the mordanting effect of the aldehyde (that is, resistance to decolorization) in the alcohols or in acid alcohols as it is to the specificity of the staining reaction. Furthermore exposure of the cells to aldehyde, which have already been stained with basic fuchsin, does not produce a mordanting effect and does not destain the cells.

It would seem that acid hydrolysis affects both the nucleus and cytoplasm, but specifically it modifies the nuclear components so that they may react both with the basic fuchsin and with the aldehyde. That the same chemical radicals are involved in the nucleus in both reactions is suggested by the fact that the mordanting effect of alde-

hyde is blocked if the stain is applied first. Both stain and mordant, however, may be washed out and each apparently replaced by the other.

It is of interest that nuclear staining following hydrolysis occurred with both methylene blue and azure I, with or without formaldehyde, but that the aldehyde produced no mordanting effect. It is equally interesting that crystal violet, another basic dye of the same series as basic fuchsin, but which differs from it only in the presence of methyl groups attached to the two para-amino groups (thus blocking the activity of these amino groups), behaves in a manner similar to methylene blue and stains, but is not mordanted in the presence of aldehyde.

This strongly suggests that the para-amino radicals in the dye are the active ones in the staining reaction and that when these are blocked with methyl groups no permanent linkage can occur between the dye and the radicals in the nucleus freed by the hydrolysis, whatever these may be.

Our statements should not be construed as implying that the addition of an aldehyde radical to a nucleus which is initially Feulgen-weak or Feulgen-negative induces a state or reaction, comparable or equivalent to a Feulgen reaction. It is suggested, however, that further study of the staining reactions described herein might be productive in studies of the Feulgen reaction.

Our observations from study of Feulgen-weak organisms give only a partial answer to the problem. Neither the Feulgen reagent under various conditions nor basic fuchsin decolorized with hydrochloric acid produced an adequate stain for cytologic study. Aldehyde-colored Feulgen reagent (Choudhuri, 1943) stained, but was not resistant to decolorization. On the basis of direct observation of the structures stained it seems likely that Choudhuri's stain, aqueous basic fuchsin and the two aldehyde mordant stains described herein, all stain the same nuclear structures. The nuclei under consideration are, however, so small that certainty in this matter cannot be absolute.

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THE EFFECT OF NUCLEASES ON CYTOCHEMICAL REACTIONS FOR AMINO ACIDS AND ON STAINING WITH ACID DYES¹

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ABSTRACT.—Crystallin ribonuclease has no marked proteolytic activity, since digestion of sections with this enzyme produces no appreciable decrease in the intensity of the cytochemical tests for arginine and tyrosine. Cytoplasmic basophilia is unaffected by treatment with cold trichloroacetic acid or with boiling alcohol-ether mixture. Mononucleotides and fatty acids thus have nothing to do with basophilia. Digestion of sections with desoxyribonuclease has no effect on the alkaline phosphatase or arginine tests, while it suppresses the Feulgen reaction and the affinity of the chromatin for basic and for some acid dyes.

INTRODUCTION

Extensive use of the enzyme ribonuclease has been made in recent years for the detection of ribonucleic acid in sections of animal tissues. One of the present writers (Brachet, 1947) found that the nucleolus and the cytoplasm lose their affinity for basic dyes (toluidine blue, or the pyronin of methyl-green-pyronin mixtures) when sections were incubated, previous to staining, with ribonuclease. This loss of stainability has been used to indicate the presence of ribonucleic acid in the nucleolus and in the basophilic cytoplasm. This conclusion seems justified when taken in conjunction with the results of Caspersson's method, which is quite different, based as it is on the strong absorption in the ultra-violet of nucleic acids. Furthermore, chemical analysis of the pentose content of various tissues shows that there is a definite correlation between the intensity of cytoplasmic basophilia and ribonucleic acid content (Brachet, 1941).

However, there have been numerous reports that crystallin ribonuclease may have proteolytic activity as well. Schultz (1941), for example, found that ribonuclease removes an acidophilic constituent of salivary chromosomes, and Mazia (1941) has made comparable observations on the same material. Using a quantitative ultra-violet spectrophotometric method, Gersh and Bodian (1943) found

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that ribonuclease strongly decreases nucleic acid absorption in neurones, while the absorption of proteins is decreased to a lesser extent. More recently, Schneider (1946) found that different preparations of ribonuclease vary in proteolytic activity. Since the latter is often negligible, while the action of the enzyme on ribonucleic acid is always very pronounced, Schneider believes that, on the whole, ribonuclease is really useful in the detection of ribonucleic acid.

In view of this somewhat confused situation, we have studied the action of ribonuclease on various tissues from a somewhat different standpoint. After treatment of sections with ribonuclease, we have, in addition to staining some with basic dyes, also applied the cytochemical tests for arginine and tyrosine, described by Serra (1946) and Thomas (1946), to other, similarly treated, sections. These two amino acids are of particular interest since, according to Cohen (1945), they are very easily split from proteins by ribonuclease. We have also compared the effect of extraction with cold and hot trichloroacetic acid on cytoplasmic basophilia. This first treatment is often used to extract mononucleotides from tissues in chemical analyses without removing nucleic acids, while the second procedure has been shown by Schneider (1946) to extract quantitatively both types of nucleic acids. Pollister and Mirsky (1947) have recently reported that treatment of sections with hot trichloroacetic acid removed all the basophilic material from the cells. Finally, a few observations on the effect of thymonucleodepolymerase (desoxyribonuclease) on the staining of sections with acid dyes, and on the arginine and alkaline phosphatase reactions, are also reported.

EXPERIMENTAL RESULTS

1) THE EFFECT OF RIBONUCLEASE ON THE ARGININE AND TYROSINE TESTS

Crystallin ribonuclease, obtained through the kindness of Dr. J. Kunitz, was used in these experiments. The enzyme was used in a concentration of 100 mg. per cent and was dissolved in either distilled water or in McIlvaine's citric acid buffer at pH 6.0 or pH 7.0. The use of this buffer has recently been advocated by Stowell and Zorzoli (1947), who studied thoroughly the influence of various factors on the action of ribonuclease on tissue sections. As it turned out, however, distilled water (at pH 6.3) was just as satisfactory a solvent for the ribonuclease as the buffer solutions, in this study. Tissues were fixed in Serra's alcohol-formol-acetic acid mixture for a short time (half an hour to an hour, depending on the size of the piece of

tissue being fixed), and then embedded and sectioned in the usual way (all sections were cut to the same thickness in this series of experiments). Mounted sections were then incubated (after deceleration) with ribonuclease for one hour at 52°C. Two sets of control sections were run simultaneously: untreated sections, and sections placed in the buffer solution or in the distilled water for one hour at 52°C.

Control and ribonuclease-treated slides were then handled, simultaneously, in the following way: stained in a saturated solution of toluidine blue followed by thorough (two treatments of 5 minutes each) differentiation in 95% ethyl alcohol; stained in Unna-Pappenheim's methyl-green-pyronin² mixture, followed by short differentiation in 95% ethyl alcohol; or subjected to tests for arginine or for tyrosine according to the methods of Serra and of Thomas (Serra's and Thomas' methods for arginine gave substantially the same results).

The tissues used were rich in ribonucleic acid, showing distinct basophilia. They included young frog oöcytes, frog gut and liver, and the eggs of sea-urchin and star-fish.

It was found, as usual, that treatment with warm water or warm buffer decreased basophilia to a variable extent, while incubation with ribonuclease produced the complete disappearance of cytoplasmic basophilia and nucleolar basophilia, without any appreciable effect on the staining of the chromatin. As for the effect of the ribonuclease treatment on the cytochemical tests for arginine and tyrosine, no difference could be seen between untreated sections, control sections and sections treated with the enzyme. Thus, complete disappearance of basophilia may occur without a visibly appreciable decrease in the intensity of the arginine and tyrosine reactions. The possibility that a slight decrease in the content of these two amino acids (appreciable only by quantitative microphotometric methods) might occur is, of course, not excluded by our observations.

2) ACTION OF TRICHLORACETIC ACID ON BASOPHILIA

When sections of frog ovary, fixed in Serra's fluid, are treated with ice-cold 5-10% trichloroacetic acid mixture for 15 to 30 minutes and stained with toluidine blue or Unna's mixture, no decrease in basophilia is observed. The cells in these sections, then, contain no acid-soluble basophilic material. Actually, the presence in fixed

²Pyronins of different manufacture often have very different staining properties; Grüber's pyronin was used in the present work.

tissues of substances of a mononucleotide nature is rather unlikely, since these substances are soluble in most fixing solutions.

On the other hand, the heating of similarly fixed tissue sections in trichloroacetic acid (5–10%) to 90°C. for 15 minutes produces the complete disappearance of all basophilic material, both in the nuclei and in the cytoplasm. As was to be expected, this treatment had no effect on the cytochemical tests for arginine and tyrosine. These results fully confirm Schneider's (1946) chemical data and the cytochemical observations of Mirsky and Pollister (1942).

A conceivable criticism of the specificity of the ribonuclease method for the detection of ribonucleic acid is the possibility that fatty acids might be responsible for cytoplasmic basophilia. This criticism has been made by Holtfreter (1946), who worked on unfixed amphibian eggs. It is, of course, unlikely that large amounts of fatty substances would remain in histological sections after prolonged treatment with alcohol and toluene. Furthermore, it has been pointed out by Monné (1946) that cells stain more strongly with basic dyes when fixatives which dissolve lipids (such as Carnoy's or Serra's fluids) are used.

In order to check the validity of Holtfreter's objection further, frog ovary sections were extracted with a boiling alcohol-ether (1:1) mixture in a Soxhlet apparatus. Such a treatment has been found successful by Cohen and Chargaff (1941) in their studies of lipids associated with the ribonucleic acid in microsomes. The sections were then stained with toluidine blue in the usual way. The thorough extraction of lipids was seen to produce no decrease at all in the affinity of basophilic material for the basic dye.

3) THE ACTION OF DESOXYRIBONUCLEASE (DNase) ON THE PHOSPHATASE AND ARGININE REACTIONS AND ON STAINABILITY WITH ACID DYES

It is a well established fact that the Gomori reaction for alkaline phosphatase is usually strongest in the chromosomes and in the nucleolus. It was, therefore, of some interest to know whether or not enzymatic removal of the desoxyribonucleic acid in the chromosomes would have any effect on the phosphatase reaction.

The DNase was prepared from beef pancreas according to the method of Fischer and his coworkers (1941), and was used in a concentration of 10 mg. per ml. Under these conditions, removal of desoxyribonucleic acid, as indicated by the disappearance of the Feulgen reaction and of the stainability of the chromatin with basic dyes, was almost instantaneous at room temperature. The enzyme, however, was active only if traces of magnesium ions were added;

the non-magnesium-activated enzyme constituted a convenient control.

If DNase is made to act on alcohol-fixed sections of mouse intestine for five minutes or less at room temperatures, it is found that the nuclei stain no longer with toluidine blue, or give a positive Feulgen reaction. The Gomori alkaline phosphatase reaction, however, remains very intense. As a matter of fact, it is doubtful that any decrease in the intensity of the phosphatase reaction occurs at all. These results are in good agreement with Jeener's findings (1946) that isolated nuclei treated with 1M NaCl, in order to remove the thymonucleohistone, still retain their alkaline phosphatase. Similar conclusions have been reached from a study of isolated chromosomes treated with 1M NaCl, by Mirsky and Ris (1947).

Staining with an acid dye, such as ponceau 2R, in an acid medium and in the presence of a detergent has been advocated by Hydén (1943) as a convenient method for the cytochemical detection of basic proteins of the histone type. When using this method on various tissues it was found that the intensity of the staining very closely resembled the picture given by a basic dye like toluidine blue. Differences in the staining pictures obtained with the two dyes, however, sometimes occur, as in the case of amphibian eggs, where the yolk platelets stain intensely with ponceau 2R, but not with toluidine blue. Yolk platelets also give an intense arginine reaction (Brachet, 1947) and are thus presumably rich in basic proteins. In view of the unusually strong staining with acid ponceau 2R, it seemed worthwhile to study the effects of pretreatment with DNase on stainability with this dye. Tissues of various organs (mouse intestine and pancreas, frog ovaries) were fixed in Carnoy's or Serra's fluids and stained according to Hydén's method, before and after treatment with DNase. Similarly handled sections were also stained simultaneously with the Feulgen technic and with toluidine blue. It should be pointed out that the ponceau 2R has not been standardized and the sample used in these experiments came from a different manufacturer than that used by Hydén (G. T. Gurr, London, Lot. No. 1044; Hydén used IGEFA ponceau 2R).

It was found that DNase always produced a very strong decrease or a complete disappearance of the staining of the nuclei, without acting on the cytoplasm. The effects of the enzyme were thus completely identical, whether the sections were stained with the Feulgen technic, with toluidine blue or with ponceau 2R. Such results might be due to two possibilities; either the ponceau 2R used stains desoxyribonucleic acid (presumably by reacting with the amino groups of the aminopurines, which are dissociated at a low pH), or the DNase

contains impurities which remove the basic proteins from the sections (proteolytic action).

In order to choose between these two alternatives, sections of the same tissues were submitted to Serra's arginine reaction prior to and after the action of DNase. In contrast to the above findings on nuclear basophilia, DNase has no effect at all on the intensity of the arginine reaction. This enzyme, in the conditions of the experiments (very short incubation times) thus had no demonstrable proteolytic activity.

The conclusion that the ponceau 2R we worked with can stain desoxyribonucleic acid thus becomes very likely. Such a conclusion finds additional support in experiments done on nucleic acid and on nucleohistone. Polymerized desoxyribonucleic acid, prepared from thymus and purified by the Hammarsten (1924) procedure, was embedded in paraffin, after reprecipitation with alcohol containing uranium acetate to prevent the dissolution of the acid in water. Sections of the precipitate were then cut and treated according to the usual histological technics. Thymonucleohistone was prepared from red blood cells by the method of Mirsky and Pollister (1942), fixed in alcohol, embedded in paraffin and sectioned. The slides were then stained with ponceau 2R, toluidine blue or submitted to the Feulgen reaction and to Serra's arginine reaction.

It was found that the pure desoxyribonucleic acid gave no positive arginine reaction; it stained, however, very strongly with ponceau 2R, as well as with toluidine blue and gave a very strong Feulgen test. In the case of thymonucleohistone, all reactions were strongly positive, but after a short incubation with DNase, the Feulgen reaction became negative and the precipitate did not stain any longer with ponceau 2R, or with toluidine blue. The arginine reaction, on the other hand, remained completely unaffected by the enzymatic digestion.

DISCUSSION OF THE RESULTS

The evidence presented above on the action of ribonuclease, of trichloroacetic acid, cold and heated to 90°C., as well as of fat solvents, all goes to show that ribonucleic acid, and not mononucleotides or fatty acids, is the substance responsible for the *orthochromatic* cytoplasmic basophilia³ in the tissues we have studied. This evidence thus lends additional support to the view presented earlier by one of us (Brachet, 1947), and by Caspersson (1941). We, of course, cannot rule out the possibility that ribonuclease might have some slight proteolytic activity, presumably due to the presence of some contaminant. In the case of the preparation we used, however,

³For a discussion of this matter, see Michaelis (1947).

there is no doubt that the action on proteins was negligible, as compared with the effect on basophilia. Such a conclusion is in good agreement with Schneider's (1946) chemical data and with the fact that Cohen (1945) had to use very long (48 hours) incubation times in order to demonstrate a proteolytic activity of ribonuclease in the case of nucleohistone. Since it has recently been shown by McDonald⁴ that crystalline ribonuclease, completely free of proteolytic activity, can still easily remove cytoplasmic and nucleolar basophilia, there seems to be little reason to doubt that the use of ribonuclease constitutes an easy and sensitive, as well as specific, test for the cytochemical detection of ribonucleic acid.

As regards the fact that the basophilia remains unaffected by treatment with cold trichloroacetic acid and boiling alcohol-ether mixture, it might be noted that the experimental conditions were unusually favorable for the extraction of mononucleotides and of fatty acids, respectively. In most biochemical methods, these extractions are performed on pieces of tissue that are much thicker than the thin sections used by us.

The proteolytic activity of DNase seems also to be negligible, at least during the short periods of incubation reported here, and use of this enzyme should prove to be a useful addition to the other methods for the cytochemical detection of desoxyribonucleic acid. The need of such a method, however, is obviously not so important as in the case of ribonucleic acid, because of the existence of the Feulgen reaction, the specificity of which has recently been reinvestigated by Stowell (1946) and by one of us (Brachet, 1946).

Finally, concerning the unexpected results obtained with staining with ponceau 2R, it seems likely that they were due to the nature of the particular dye used in these experiments, because the ponceau 2R used by Hydén did not stain pure desoxyribonucleic acid (personal communication of Dr. Hydén). It seems advisable, when Hyden's method is used with an unstandardized acid dye of unknown properties, to first check whether the dye stains desoxyribonucleic acid or not. This can easily be done by treating sections with DNase and following the effects of the enzymatic digestion with both the Feulgen reaction and with Hydén's acid dye technic.

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⁴See discussion following paper by Brachet (1948).

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SAFRANIN AND ANILIN BLUE WITH DELAFIELD'S HEMATOXYLIN FOR STAINING CELL WALLS IN SHOOT APEXES¹

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ABSTRACT.—The following technic is suggested for staining cell walls in shoot apices: After the usual preliminary steps through 50% ethyl alcohol, stain in 1% safranin O for 24 hours. Rinse in tap water and place in 2% aqueous tannic acid for 2 minutes. After rinsing in tap water, stain for 2 minutes in 1 part Delafield's hematoxylin to 2 parts distilled water and rinse in tap water. Remove excess hematoxylin with acidified water (1 drop conc. HCl in 200 ml. water), then place slides in 0.5% lithium carbonate for 5 minutes. Dehydrate through an ethyl alcohol series, then transfer from absolute alcohol to a saturated solution of anilin blue in "methyl cellosolve" for 5–10 minutes. Wash in absolute alcohol, rinse in a solution of 25% methyl salicylate, 33% xylene, 42% absolute ethyl alcohol and clear for 10 minutes in a solution of 2 parts methyl salicylate, 1 part xylene, 1 part absolute ethyl alcohol. Transfer through two changes of xylene and mount in "clarite" or suitable alternate. The resulting preparations will have clearly defined, dark-staining cell walls and will photograph well when "Super Panchro-Press, Type B" film (Eastman Kodak Co.) is used in conjunction with suitable Wratten filters.

INTRODUCTION

In recent years increasing emphasis has been upon the developmental phases of plant anatomy. Numerous papers have appeared which record the organization of the apical meristem and the development of foliar organs. It is understandable then, that some new technics for staining the small, thin-walled cells of the meristem have been developed and made available to other investigators working in the field. The urgent need for good staining methods for the shoot meristem does not become apparent until an attempt is made to prepare material for study. It then becomes evident to the technician that the chemistry of the cell components of the apical meristem is considerably different from that of more mature cells. The most disturbing fact, however, is that the cell contents

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and the cell walls of the meristem often do not stain differentially when treated with usual stain combinations. In attempting to stain stem tips with safranin-fast-green, it frequently happens that the safranin stains the nucleus, cytoplasm, and cell walls with equal intensity. It is difficult in many cases to stain any of these parts very sharply. In the process of either intentional or unintentional decolorization, the safranin is removed from all structures with equal rapidity. The fast green dye acts in approximately the same way in many instances. The results are often far from satisfactory; all cell parts appear various shades of reddish-violet with none sharply defined. The outcome is a cloudy preparation which is difficult to study and to photograph.

It should not be inferred from the foregoing that safranin-fast-green is worthless as a stain for apical meristems. Our experience has been, however, that the chances of this combination giving superior results on angiosperm material are rather slight. Similarly, other stain combinations seem to give good results when applied to some plant materials (or in the hands of some technicians) while in other cases they result in unusable preparations. Certain of the stain combinations which have been proposed most recently and which have yielded excellent results in some instances should be mentioned.

Sharman's (1943) combination of tannic acid with iron alum, safranin and orange G has been used with success by the author on shoot apices and other tissues of grasses.

Boke (1939) has obtained satisfactory results using safranin and Delafield's hematoxylin for shoot apices of *Acacia*, *Trichocereus*, *Opuntia*, *Tradescantia*, *Ginkgo*, and *Zamia*. Ball employed this schedule in his studies on *Phoenix* and Hsu used it for *Sinocalamus*.

Cross' (1937) safranin-fast-green procedure has been used in preparing slides for histogenic studies on *Taxodium*, *Sequoia*, *Athrotaxis*, *Taiwania*, *Cunninghamia*, *Cryptomeria*, *Viburnum*, and *Vinca*.

Foster's (1934) schedule with tannic acid and iron chloride and modifications of it have been rather generally attempted by various investigators and successfully used for *Pseudotsuga* (Allen; Sterling), various cycads (Johnson), *Sequoia* (Sterling), *Garrya* (Reeve), and *Amygdalus* (Brooks). The method, developed during a study on *Carya*, was later used by Foster for *Rhododendron*, *Ginkgo*, *Cycas*, and *Microcycas*.

Some of these schedules are rather long, tedious, and seem to require a rather high degree of luck or skill which must be learned by trial and error. For instance, some very excellent technicians have abandoned Foster's method after many unsuccessful attempts to use it. We are convinced that these failures are not often due to poorly

written instructions or to slovenly technic but rather to the variability of the material itself. Some indications of this variability may be inferred from a study of the list of genera following each schedule mentioned above. It appears that the proper selection of a stain schedule is of more than usual importance to the student of apical meristems.

The schedule set forth below, therefore, is not offered as a fool-proof method for all shoot apices but rather as another method which has been used with satisfactory results on *Coleus*, *Oleander*, *Chrysanthemum*, *Dracaena*, *Pinus* and other genera. Perhaps it is a technic

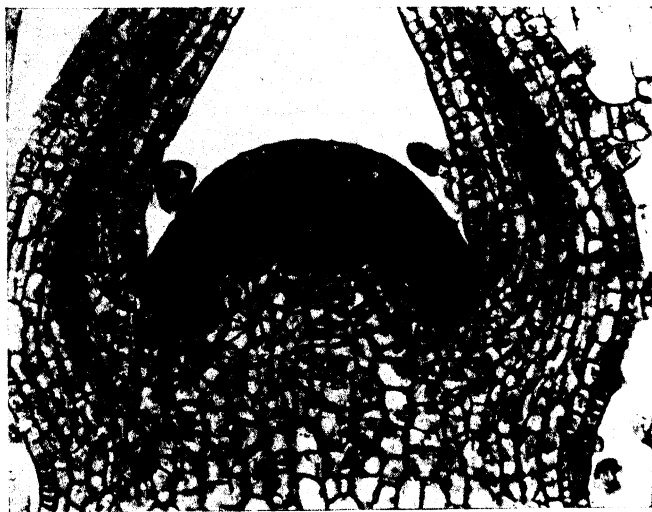


FIG. 1.—Median longisection of *Coleus blumei* stem apex cut at 6μ , stained by the schedule omitting steps 4, 5, 6, & 7, and photographed on Eastman "Super Panchro-Press, Type B" film using a Wratten A filter number 25. $\times 280$.

which may frequently or usually be expected to give good results. In any event, the schedule is rather short and simple in that the timing factor does not seem to be extremely critical.

STAINING SCHEDULE

Every technician is well acquainted with the fact that most schedules are provisional and must be modified for each different type of plant material. The following is no exception:

1. Bring slides to 50% ethyl alcohol.
2. Stain in 1% safranin O for 24 hours.

3. Dip² in tap water.
- 3a. Dip in 2% aqueous tannic acid solution and leave for 2 minutes.
- 3b. Dip in tap water.
4. Dip in Delafield's hematoxylin (1 part hematoxylin diluted with 3 parts distilled water) and leave for 2 minutes.
5. Dip in tap water.
6. Dip in acidified water (1 drop conc. HCl in 200 ml. water) to remove excess hematoxylin.
7. Dip in 0.5% lithium carbonate in distilled water and leave for 5 minutes or wash in running alkaline tap water for 5-10 minutes.
8. Dip in each of the following: 50%, 70%, 95%, and absolute ethyl alcohol. If an excess of safranin remains in the sections, a quick dip in 200 ml. 95% ethyl alcohol to which 1 drop of HCl has been added may be used immediately following the 70% alcohol.
9. Dip in a saturated solution of anilin blue in "methyl cellosolve" and leave for 5-10 minutes.
10. Dip in absolute ethyl alcohol to remove excess anilin blue from the sections and from the slide.
11. Dip in a rinse solution (25% methyl salicylate, 33% xylene, 42% absolute ethyl alcohol).
12. Dip in a clearing solution (2 parts methyl salicylate, 1 part xylene, 1 part absolute ethyl alcohol) and leave for 10 minutes.
13. Dip in a mixture of 90% xylene and 10% absolute ethyl alcohol.
14. Dip successively into two Stender dishes of xylene and leave until ready to proceed.
15. Mount in "Clarite" or suitable alternate.
16. Cure slides in 40°C. oven for 2 weeks or more.

Although no attempt was made to determine the particular type of killing fluid which would give the best results, FPA (5 ml. formalin, 5 ml. propionic acid, 90 ml. 50% ethyl alcohol) has always given satisfactory fixation. The vacuum apparatus described by Wittlake (1942) was used to remove trapped air from the stem tips and to speed penetration of the tissues by the killing solution. Following the killing fluid, the material was dehydrated with an ethyl alcohol series. Toluene was employed as the rubber-paraffin vehicle.

²Whenever the term "dip" appears in the schedule, it should be interpreted to mean "raise and lower the slide rapidly 10 or 12 times". Sharman (1943) recently called attention to the fact that this procedure insures quick replacement of the old solution with the new and thus enables one to move slides through the series more rapidly.

Longitudinal and cross sections of shoot apices were cut at thicknesses varying from 4μ to 8μ .

The most satisfactory solution used in the schedule was prepared as suggested by Johansen (1940) by dissolving 4 g. of safranin O (National Aniline and Chemical Co.) in 200 ml. "methyl cellosolve" (ethylene glycol mono-methyl ether) (Carbide and Carbon Chemicals Corp.) then adding 8 ml. formalin, 4 grams sodium acetate, and 200 ml. 50% ethyl alcohol. Aqueous or alcoholic solutions of safranin give definitely inferior results. Steps 3a and 3b may usually be omitted without impairing the results. In staining some plant



FIG. 2.—Median longitudinal section of *Chrysanthemum* hybrid stem apex cut at 8μ , stained by the complete schedule, and photographed on Eastman "Super Panchro-Press, Type B" film using a Wratten G filter number 15 and a Wratten K-2 filter number 8 in series. $\times 200$.

materials it is felt that the tannic acid acts as a mordant for safranin resulting in a more brilliant stain and less rapid decolorization in the alcohols. Fine preparations may usually be obtained by a safranin-anilin-blue stain combination in which steps 4, 5, 6, and 7 are omitted from the proposed schedule (Fig. 1) but their inclusion has consistently given better results (Fig. 2). The thin cell walls stain more deeply purple and are more sharply differentiated. It has been our experience that safranin and Delafield's hematoxylin seldom result in preparations with sharply defined cell walls.

The success or failure of the procedure is in large measure dependent upon the anilin blue solution used. A dye manufactured by Coleman and Bell Co., designated as "Aniline Blue, for use in histological work, especially in Mallory connective tissue stain" gave superlative results. A saturated or more dilute solution of this dye dissolved in "methyl cellosolve" may be used depending upon the material to be stained. Since the dye used was from batches CK-3 and CK-4 certified by the Biological Stain Commission, it is probable that satisfactory results will be obtained with dye from other similarly certified batches. The uncertified anilin blue dyes designated "water soluble" which were tested, were found to be worthless and should be avoided in making up stain solutions. It is possible that the infrequent employment of anilin blue stain by botanists has been due largely to the poor results obtained when an unsatisfactory batch of dye and an inferior solvent were used.

In the transverse and longitudinal sections of apical meristems cut 4μ to 8μ thick and stained by this schedule, the cell walls appear dark purple and sharply defined, the cytoplasm stains purple, the nuclei reddish purple, and the nucleoli and chromosomes a brilliant red. Procambium cells, phloem elements, and cells of other tissues in older portions of the shoot tip appear clearly defined.

Fastness of the dye to light is not usually a prime consideration in selecting a stain for research purposes. Although the slides prepared were not submitted to rigorous tests, no noticeable fading has occurred after more than 2 years.

Satisfactory photomicrographs (Fig. 1, 2) have been obtained by using "Super Panchro-Press, Type B" film (Eastman Kodak Co.) and suitable Wratten filters. If the complete schedule is used, a combination of a Wratten G filter number 15 and a Wratten K-2 filter number 8 in series gives sharp negatives (Fig. 2). If steps 4, 5, 6, & 7 are omitted from the schedule, a Wratten A filter number 25 gives good results (Fig. 1).

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STAINING SECTIONS OF PERIPHERAL NERVES FOR AXIS CYLINDERS AND FOR MYELIN SHEATHS

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ABSTRACT.—Pieces of mammalian nerves 1 to 2 cm. long were placed under moderate tension and fixed 24–48 hours in: picric acid, saturated aqueous, 90 ml.; formalin, 10 ml.; and trichloroacetic acid, 25% aqueous, 2 ml. They were washed in water, cut in two and one end stained with 0.04–0.06% osmic acid solution, while the other was dehydrated, embedded in paraffin, and mounted sections from it stained with protargol. The fixing solution used was selected from a number of combinations of acidified picroformalin as the one most likely to give satisfactory results when followed by both silver and osmic acid. The use of osmic acid solutions of less than 0.1% concentration avoided the overstaining of myelin sheaths seen frequently when stronger solutions were used with material that had been fixed previously. Protargol, 0.5% solution with fast green FCF added to make 0.05% dye in the final concentration, was used to impregnate sections for axis cylinders. Reduction and toning were done as in Bodian's method.

Studies of peripheral nerves which involve numbers and sizes of fibers or ratios of myelinated to unmyelinated fibers require a staining technic which yields a high percentage of usable results. The procedure used should enable one to secure cross sections stained specifically for axis cylinders and for myelin sheaths and such sections should come from closely adjacent portions of the nerve to be studied.

Three types of procedures that have been used in this and in other laboratories may be mentioned. (1) The technic used by Bruesch and Arey (1940) on optic nerves was as follows. The fresh nerve was cut in two at the site where the sections were to be taken. One portion was fixed in osmic acid vapor while the other was fixed in formalin for subsequent staining with silver on the slide. (2) A more direct procedure, which would show axons and sheaths on the same section has been developed by Foley (1943). Frozen or celloidin sections were stained with protargol and counterstained for myelin sheaths with dyes. (3) The method that has been used most frequently in this laboratory consists of first fixing the nerve specimen,

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cutting in two at the site where sections are desired, and then processing one piece for axis cylinders and the other for myelin sheaths. The present status of this method, based upon recent experimental work, will be described.

SOURCES OF NERVES

Human genitofemoral, dog sciatic and vagus, and rat sciatic and common peroneal nerves were used. The human and dog nerves were normal, but both normal and regenerating nerves of rat were included.

FIXATION

Since fixation must be done in aqueous solution to preserve myelin, all fixing solutions were made with distilled water. The following comments cover our observations.

1. *Formalin alone.* Shrinkage and distortion of myelin sheaths were objectionable and subsequent staining by protargol was weak.

2. *Formamide and trichloroacetic combinations.* Shrinkage of axis cylinders was excessive, myelin sheaths were polygonal, crenated or oval in cross section, and the myelin had a tendency to clump within the sheath. Protargol stained strongly, however.

3. *Picric acid.* This reagent penetrated rather slowly when used alone, but very small nerves fixed adequately. It was not deleterious to subsequent staining with either silver or osmic acid. Little or no consistent improvement was accomplished by mixing it with acetic, trichloroacetic or formic acid (without formalin). When mixed with

PLATE 1

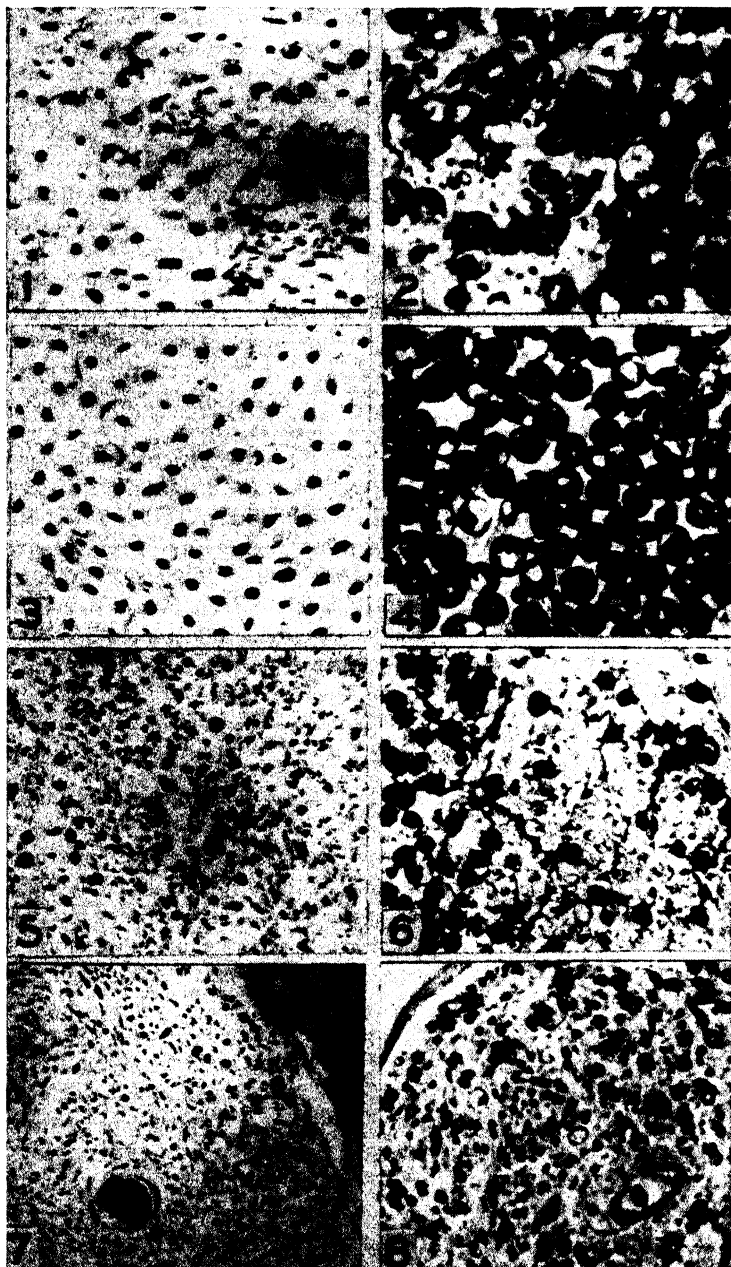
Representative areas of mammalian nerves stained by silver and by osmic acid after fixation in a modified Bouin's fluid. The sections were secured within about 1 mm. of each other.

FIG. 1 and 2.—Human sensory (genitofemoral branch). A considerable number of the fibers are not myelinated, as is shown by the abundance of small fibers in the silver stain.

FIG. 3 and 4.—Motor component of the tibial nerve of dog. There are scarcely any unmyelinated fibers here.

FIG. 5 and 6.—Vagus of dog. The unmyelinated fibers predominate.

FIG. 7 and 8.—Regenerating common peroneal of rat. Unmyelinated, recently re-grown fibers predominate. Many of the very small myelinated fibers seen in 8 will become larger as regeneration progresses.



formalin, without additional acid, fixation was good and the tendency toward weak staining by protargol (after formalin alone) was partially corrected.

4. *Formalin with acid.* Addition of 0.5 to 1.0% formic acid to 10% formalin usually improved both preservation and staining of myelin sheaths. Likewise, the addition of 0.2 to 0.5% trichloroacetic or both trichloroacetic and formic acids effected improvement over plain formalin. The subsequent staining of axis cylinders was rather erratic.

5. *Acidified formalin with picric acid* (Bouin's). Various combinations of formalin-picric with formic acid and trichloroacetic acid substituted for acetic as in the original Bouin's formula were tried. Control specimens fixed with regular Bouin's fluid were run for comparison. Large amounts (over 1%) of these substitutes for acetic acid were judged unfavorably. The most satisfactory fixation was obtained when acetic acid was replaced by 0.5% trichloroacetic acid in the usual 10% formalin in saturated picric acid solution. Tissues fixed in this mixture gave the best compromise in staining by both silver and osmic acid, and in addition did not show the excessive swelling of connective tissue seen after regular Bouin's. Although 0.5% trichloroacetic may seem to be a low concentration, it contributes many more hydrogen ions to the fixing fluid than 5% acetic. It is inherently a very efficient protein precipitant, hence causes a minimum amount of distortion of connective tissue.

STAINING

Bodian's protargol method, with suitable modifications, was used for axis cylinders. The use of metallic copper in the protargol solution was deleterious when nerves had been fixed in picric acid mixtures and, after a few trials, was omitted. It was found also that 0.5% protargol without copper was adequately strong. A staining mixture of 0.5% protargol and 0.05% fast green FCF (reported previously, Davenport, Porter and Thomas, 1947) was used for most of the preparations.

The first attempts to obtain stains with osmic acid on the portion of the nerve allocated for myelin sheaths were made by using 0.25% osmic acid. Small nerves were grossly overstained and larger ones stained too dark on the periphery. Dilutions of osmic acid between 0.025 and 0.06% were then tried, with much more satisfactory results. When the nerve is already fixed, it appears that a much lower concentration of osmic acid is required for staining than that required when it is being used as a combined fixing and staining agent.

TECHNIC RECOMMENDED

1. The piece of nerve is freed from adherent fat by careful dissection without trauma, and each end tied with a short length of No. 60 sewing thread. The specimen is then suspended on a narrow strip of cardboard which has been bowed to exert some tension on the nerve and to allow free access of the fixing fluid all around it. Slits can be cut in the ends of the card and the threads pulled through. These will usually hold without tying, but the threads may be tied across the convex side of the bowed strip if necessary.

2. Fix about 24 hr. in: Formalin, 10 ml.; Saturated aqueous picric acid solution, 90 ml.; 25% aqueous trichloroacetic acid solution, 2 ml.

3. Wash 0.5 hr. in distilled water.

4. Remove the specimen from the card by cutting the threads. Care should be taken to avoid bending the nerve.

5. Place the nerve on a piece of cork and cut in two at the site where sections are desired. A sharp, clean safety razor blade can be used. The nerve is held by placing one tip of a pair of forceps against it and using the tip as a guide for the razor blade. Avoid squeezing the nerve. The pieces are designated A and B for convenience in following the subsequent processing.

5A. Transfer the end to be stained with silver to 70% alcohol.

6A. Complete the dehydration in graded alcohols, clear, and embed in paraffin.

7A. Cut 6–10 μ sections, mount on slides and stain with protargol. Bodian's (1936) method can be used, but should be modified by omitting the metallic copper from the staining solution. The details of the fast-green-protargol method (another modification) have been published previously (Davenport, Porter and Thomas, 1947).

5B. Transfer the end to be used for osmic acid staining to distilled water and wash through 2 or 3 changes of 0.5 hr. each.

6B. Place in 0.04 to 0.06% osmic acid solution. Very small nerves stain with the weaker solution.

7B. Allow to stain for about 24 hrs. and wash well in distilled or running tap water.

8B. Dehydrate, embed in paraffin, and cut and mount sections in the usual manner.

Caution.—When cutting these specimens, be sure to save the sections from the very beginning of the block. Penetration of osmic acid is limited to 1 mm. or less, and the best sections may occur near the beginning of the series.

A few millimeters of thread should be left on the ends originally tied and used for handling with forceps. The cut pieces are embedded with the cut ends "out" in the paraffin blocks. The silver and osmic sections will be mirror images unless one or the other is turned over

at the time of placing on the slide. The section stained with silver will be smaller in area, as a rule, because less shrinkage occurs in the specimen treated with osmic acid before embedding.

Regenerating nerves have given good results more consistently than normal ones, and small nerves may be expected to respond better than large ones.

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A BUFFERED GIEMSA-BODIAN STAIN FOR NEUROLOGICAL MATERIAL

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ABSTRACT.—A buffered Giemsa counterstain for the Bodian method is described. It is very useful for bringing out Nissl substance and nerve fibers in the same section. Bouin perfused or formalin fixed material from mammals, amphibia, reptiles and fish was used. After fixation, all tissues were decalcified for at least a week in 50% formic acid (1 part) and 20% sodium citrate (1 part). This was washed out thoroughly. The method described by Bodian (1936) was followed except for the following minor changes: Winthrop Protargol (Strong Protein Silver) Batch N346BJ was used exclusively; all glassware was cleaned with acid prior to setting up the stain; before developing, the sections were washed in warm tap water for 10 minutes; the gold chloride was chilled before use. The sections were then put into buffered Giemsa for 24 hours. Stock Giemsa: 0.75 grams powdered Giemsa (Coleman and Bell, Certification No. CGe-3) was dissolved in 50 ml. of glycerin overnight in the oven, then 50 ml. of methyl absolute alcohol were added. To 3 ml. of Giemsa stock solution, 87 ml. of distilled water buffered to pH 5.3 with 10 ml. of Sørensen's buffers were added and the solution filtered. Coleman buffer tablets gave best results at pH 5.0. Sections were then rinsed in 95% alcohol, two changes of absolute alcohol, two changes of xylene, and were then mounted in Clarite.

It has been found that buffered Giemsa is a useful counterstain for the Bodian method, because of its flexibility, transparency, and the variety of structures stained. The method is especially helpful because it brings out the Nissl substance and nerve fibers on the same section, and provides a pleasing general histological differentiation (see accompanying illustration). While a variety of counterstains and combined stains for nerve fiber impregnations have been used in the past (Bodian, 1937, used acridine red; Mallory, Mallory-Azan; Foley, 1938, fast green, light green, and orange G as counterstains while Davenport *et al.*, 1947, used anilin blue, hematoxylin, gallocyanin, phloxine B, and orange G as combined stains), we feel that the method described here is both simple and reliable and has certain advantages. Buffered Giemsa-Bodian has been used in

this laboratory with equally good results on material from mammals, amphibia, reptiles and fish.

METHOD

I. PRELIMINARY STEPS

1. The animals were usually perfused with Bouin, although fresh material submerged in either Bouin or in 10% formalin also stained well. The Bouin's fixative was washed out with alcohol and ammoniacal alcohol.

2. All of our specimens were allowed to stand for at least a week in decalcifying solution to remove all traces of calcium. Calcium may be precipitated by Protargol, see Dempsey and Wislocki (1946) and Dempsey, Bunting and Wislocki (1947). We used the following decalcifying solution and washed it out very carefully with running water:

50% formic acid, 1 part
20% sodium citrate, 1 part

3. Only Winthrop Protargol especially tested for tissue staining was found to be satisfactory ("Strong Protein Silver," Batch N346-BJ, was used).

4. All glassware for the silver impregnations was carefully cleaned with nitric acid before use.

II. STAINING

Paraffin sections were treated in the way described by Bodian (1936) except for a few minor changes:

1. Before developing, the sections were washed in 2 rinses of distilled water. After developing, they were washed in warm tap water (2 changes) for at least 10 minutes, then rinsed in distilled water. This step is important to avoid contamination of the gold with developer.

We have found that two changes of tepid tap water insure better washing of the sections than running water. Running water may wash the sections from the slide if used carelessly and, since we have endeavored to make the method as fool-proof as possible, we feel that it is less suitable than warm water for 10 minutes.

2. The gold chloride which we used was 0.5% strength rather than the 1% called for by Bodian. This was for purposes of economy and worked well.

Various concentrations of gold chloride have been tried and that given (0.5%) seems to be the best compromise. As sections are passed through the gold solution it gradually becomes exhausted and eventually ceases to stain. If a large series of tissues is to be run, it is best not to start with too dilute a solution. With no concentration at room temperature did we get such sharp stains as with chilled 0.5% gold chloride.

3. The gold chloride was chilled before toning, because this seemed to inhibit the staining of the reticulum.

III. COUNTERSTAINING

1. After the silver impregnation the sections were washed in distilled water and placed in dilute Giemsa for 24 hours.

Stock Giemsa Stain. 0.75 grams of powdered Giemsa (Coleman and Bell Co., Norwood, Ohio, Certification No. CGe-3) was dissolved in 50 ml. of glycerin overnight in the oven. Then 50 ml. absolute methyl alcohol were added.

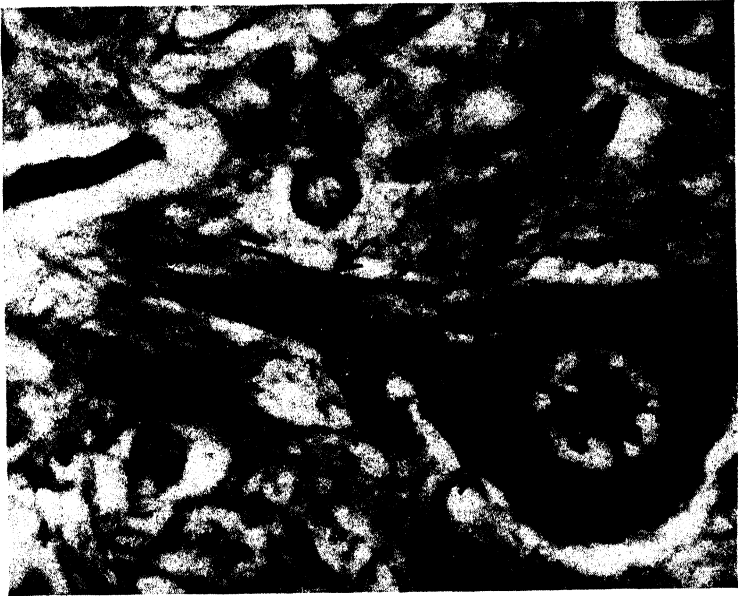


FIG. 1.—An adult rabbit motor cell showing Nissl substance and neurofibrils stained with Bodian Giemsa

To 3 ml. of Giemsa stock solution, 87 ml. distilled water buffered to pH 5.3 with 10 ml. of Sørensen's buffers (Cowdry, 1943), were added, and the solution filtered. Coleman buffer tablets can be used; however, with these the method seems to give best results at pH 5.0. It is possible and sometimes useful to vary the pH of the solution. The more alkaline the solution, the bluer the stain; the more acid the solution, the pinker the stain. In this way the stain may be adapted to different tissues or special results may be achieved.

2. Sections were then rinsed well in 95% alcohol.

3. They were next dehydrated in 2 changes of absolute alcohol, cleared in xylene, and mounted in Clarite.

RESULTS

1. Axis cylinders—black
2. Nissl substance—blue
3. Nerve cell nuclei—black
4. Glial nuclei—purple
5. Myelinated areas tend to be pink and non-medullated areas blue.
6. This also gives satisfactory definition of the cell types of the pituitary.

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ACETIC ACID METHODS FOR CHROMOSOME STUDIES AT PROPHASE AND METAPHASE IN MERISTEMS

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ABSTRACT.—Monobromobenzene and monobromonaphthalene proved easier to handle than paradichlorobenzene and acenaphthene, and at least as effective for inhibiting spindle formation, for shortening and straightening chromosomes to permit accurate counts and size comparisons. For prophase studies, methyl alcohol pretreatment was found effective in revealing centromeres, heterochromatin, and knobs. The following schedules were found simple and effective.

For metaphase chromosomes: (1) Remove root tip from germinating seed and place in saturated aqueous monobromonaphthalene for three hours. (2) Pour solution from vial and replace with a mixture of 70 volumes 95% ethanol and 30 volumes glacial acetic acid. Leave in fixative at least two days. (3) Remove opaque tip from root and macerate in a drop of acetic-orcein. Cover, heat to near boiling, flatten by pressing slide, cover down, onto a thick blotter.

For prophase chromosomes: (1) Remove root tip from germinating seed and place in 3% aqueous methanol for three hours. (2) Pour solution from vial and replace with a mixture of 65 volumes methanol, 5 of chloroform, and 30 of glacial acetic acid. Leave in fixative at least two days. (3) Remove opaque tip from root and macerate in a drop of acetic lacmoid. Cover, heat to near boiling, flatten by pressing slide, cover down, onto a thick blotter.

To develop a rapid and dependable technic for studying and counting somatic chromosomes an extensive series of substances was tested for pre-treatments of maize root tips. The series included benzene, naphthalene, all available halogen benzenes and halogen naphthalenes, anthracene, phenol, hydroquinine, phloroglucinol, and many related aromatic compounds, various alcohols and their isomers, chloroform, chloral hydrate, and other aliphatic compounds. Some of these substances had been reported by Östergren (1944) to have effects similar to colchicine. Colchicine was recommended for studies of gross chromosome morphology by the writer (1939). Swanson (1940) used acenaphthene to facilitate chromosome studies in generative nuclei of *Tradescantia*. Meyer (1945) used paradichlorobenzene on the chromosomes of *Parthenium* and other genera.

The aromatic compounds were tested as saturated solutions in

distilled water. Because most of them are extremely insoluble in water, the concentrations in many instances were lower than 1 part in 10,000. The solutions were maintained at saturation by having an excess of solute resting on the bottoms of the flasks, or floating on top when the compound was lighter than water. The required amount of solution could easily be decanted off the top, or from under the surface layer, as needed. Since very little contamination could inactivate the solution a continuous supply of solute should be kept exposed to water.

All the benzene and naphthalene compounds tested, except benzene itself (which was too strong in a saturated aqueous solution) produced the desired effect—shortened chromosomes which lay at random in the cell, owing to the absence of a spindle when the chromosomes were in the metaphase stage of contraction and thickening. The effect is apparently identical with that produced by colchicine, which was tested periodically for comparison during the trials, except that very few tetraploid cells were discovered. Of all the substances used, those which seemed most dependable in producing short, well-scattered chromosomes, were monobromonaphthalene and monobromobenzene. These substances are liquids at ordinary temperatures and are, therefore, easier to handle than paradichlorobenzene and most of the heavier halogen benzenes and halogen naphthalenes, which are solids at usable temperatures, and never form a clear solution unless carefully melted into a solid cake under the water. The following technic represents the minimum number of steps which seem possible in securing chromosome counts. When used on *Zea* (generally difficult material at mitosis), it produced slides with many metaphases which could easily be counted accurately, and many prophase, also, in which reliable counts could be made. The procedure is:

1. Remove root tip from seed and place in saturated aqueous solution of monobromonaphthalene for 3 hours.

2. Pour solution from vial and replace with a fixative consisting of 70 parts ethanol and 30 parts glacial acetic acid. Leave in fixative 2-3 days.

3. Remove opaque tip from root and macerate on a slide in a drop of acetic-orcein with the edge of another slide. Cover, heat to near boiling, flatten by pressing slide, cover down, on a thick blotter.

The preceding technic was used successfully on *Impatiens*, *Crepis*, *Lotus*, and *Avena*, as well as on *Zea*.

For detailed chromosome study at mitosis, especially at prophase, the benzenes and naphthalenes did not reveal such important details as the positions of knobs, centromeres, and heterochromatin other

than that in knobs. These details were well preserved and revealed by alcohol pretreatments, the best solutions consisting of methanol in distilled water. The effective concentrations tried were those between 1 and 7%, the best solutions being those around 3%. Material other than *Zea* may require different concentrations, but these should serve as useful first approximations. The schedule used is nearly identical with that used for metaphase counts, except that the stain and fixative are different. The standard fixative of 70 parts ethanol and 30 parts glacial acetic acid produced good results, but more detail was observable under the conditions of these trials with a fixative containing methanol and chloroform. Lacmoid was superior to carmine or orcein in staining. The schedule is as follows:

1. Remove root tip from germinating seed and place in a 3% aqueous solution of methanol for three hours.
2. Pour solution from vial and replace with a fixative consisting of 65 parts methanol, 5 parts chloroform, and 30 parts glacial acetic acid. Leave in fixative 2-3 days.
3. Remove opaque tip from root and macerate in a drop of acetic lacmoid on a slide. Cover, heat to near boiling, flatten by pressing slide, cover down, on a thick blotter.

A few precautions are necessary in making the slides by either method. The drop of acetic stain should be of such a size that when the cover is placed on it and flattened, almost no excess of stain comes out from under the cover. The ideal amount seems to be that which will leave a very narrow incomplete outline of the cover on the blotter. If an excess of stain is used, most of the free cells, which generally are those of most value, will be lost with the excess stain. A small aperture on the dropper will help in securing a drop of the correct size. The degree of heating is not critical. If the slide is too hot to be borne on the wrist, but has not boiled, it is generally well cleared. Boiling generally damages the preparation. Some selectivity of stain with different species was observed during the trials. No material was found, however, which did not stain well with one of the standard acetic acid stains—carmine, orcein, or lacmoid. Acetic lacmoid is not stable, and must be used soon after it has been prepared, preferably within ten days. The method of preparation developed by La Cour (1947) should be used with lacmoid. Unfortunately, different lots of lacmoid differ very much in staining ability, and even in gross physical appearance, and some lots are not so good as poor carmine. Orcein was found to be the next best stain when good lacmoid was unobtainable.

The preceding schedules undoubtedly can be modified to advantage for other material. They did, however, produce satisfactory results

in their present form when used on several unrelated genera, and may be adequate as they stand when only chromosome counts or idiograms are of special interest.

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MAKING DRY PREPARATIONS OF VERTEBRATE MATERIAL

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ABSTRACT.—A method is presented by which whole embryos, immature animals, parts of organs, or even thick sections, of vertebrate material are very slowly dehydrated, thoroughly cleared, and then dried to show the topography of complicated structures.

A representative photomicrograph is included to show the result obtained.

So far as I am aware, no method for making dry preparations of vertebrate material has been published yet. The method which is described here may be successfully applied on a wide variety of material such as: (a) Embryos of cartilaginous fishes to show the formation of the mouth, gill-slits, etc. (b) Amphibian eggs, morulae, gastrulae, embryos and tadpoles to show cleavage, neurulation, development of gill-slits, external gills, etc. (c) Chick-embryos to show the formation of the primitive streak, medullary tube, myotomes, gill-slits, amnion and chorion, feather-germs, formation of the beak, development of the fore- and hind-limbs, etc.

In general this method depends on prolonged, but perfect dehydration and elimination of fat, in order to prevent undue shrinkage of the tissues.

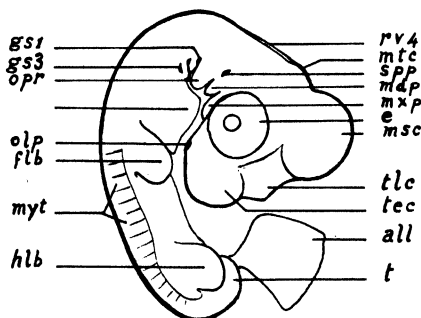
For material not more than 2 mm. in thickness, the procedure is as follows:

- (1) The already fixed material is brought to 70% alcohol. Care should be taken at this stage to clean the material from dust-particles, yolk-granules, or any foreign object, and, when necessary to remove some tissues so as to expose others.
- (2) The material is then transferred to 80% alcohol and is left for 24 hours.
- (3) Alcohol, 90%, 24 hours.
- (4) Alcohol, 96%, three changes, each for 4 to 12 hours (depending on the thickness of the material).
- (5) Absolute alcohol, three changes, each for 4 to 12 hours.
- (6) A mixture of equal parts of absolute alcohol and xylene, overnight.
- (7) Pure xylene, three changes, each for 4 to 12 hours.
- (8) The material should be left finally for at least two days in xylene.

- (9) The xylene is then drained off and the material is left partly exposed to the air so as to allow slow evaporation.

If shrinkage does take place, it is almost always too slight to cause any noticeable distortion. The surface of the material is left unchanged, and allows a good view of details when examined with the aid of a binocular microscope.

To keep a dry preparation permanent, it can be mounted on a slide in the following way: A small piece of black paper is firmly attached to a slide by means of sealing-wax or "Durofix". On this piece of paper, the material is fixed in the desired position with a minute droplet of "Seccotine" or "Durofix". A glass-ring, with a circular cover-slip already attached to it, can be fixed over the whole prep-



A photomicrograph of a dry preparation of a chick-embryo after four days of incubation. The amnion and chorion were removed.

all, allantois; *e*, eye; *flb*, fore-limb bud; *gs1*, first gill-slit; *gs3*, third gill-slit; *hlb*, hind-limb bud; *mdp*, mandibular process; *msc*, mesencephalon; *mtc*, metencephalon; *mxp*, maxillary process; *myt*, myotomes; *olp*, olfactory pit; *opr*, operculum (hyoid arch); *rv4*, roof of fourth ventricle; *spp*, spiracular pouch; *t*, tail; *tec*, telencephalon; *tlc*, thalamencephalon.

aration by means of Canada balsam, thus protecting the material from outside injuries and from dust. The piece of black paper provides a dark background on which the material shows up well by contrast (see the accompanying photomicrograph of a chick-embryo after four days of incubation).

For material more than 2 mm. in thickness, it is advisable to make a cut in the surface which is intended to be attached to the slide,

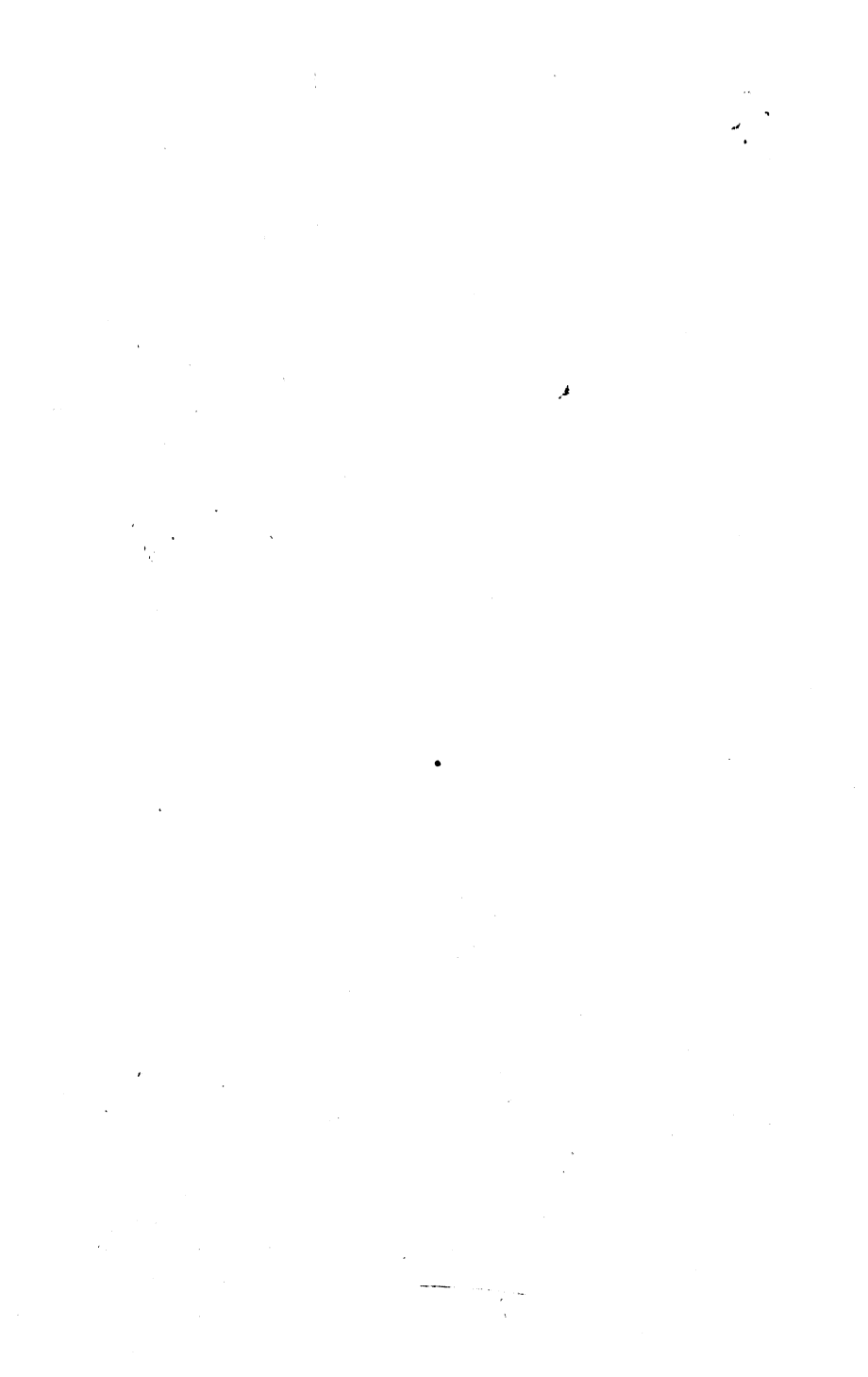
so as to allow the liquids to pass freely during dehydration, clearing, and drying.

Thick sections can be treated in a similar way.

Dry preparations can be used instead of models for class purposes. They are by far superior to models in many ways: (a) They are real, and generally look much better than any model. (b) They can be easily prepared in any laboratory. (c) They are easily portable.

I was first introduced to this method while working under the supervision of Prof. Adolf Naef (Visiting Prof. in the Department of Zoology, Fouad I University, Cairo)¹, who first applied it on Cephalopods, a reference of which is found in his monograph.

¹See NAEF, A. 1923. *Monographia die Cephalopoden*, 35, 806.



A MODIFICATION OF ALIZARIN RED S TECHNIC FOR DEMONSTRATING BONE FORMATION

METHODS OF MOUNTING, PHOTOGRAPHING AND DEMONSTRATING THE SPECIMENS

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ABSTRACT.—This paper shows that by using solutions heated in the incubator during certain stages, the alizarin red S method of staining the ossified centers in embryos has been shortened, with a consequent saving in time.

New methods of mounting the specimens have been evolved and are described in detail.

The technic of photographing mounted and unmounted specimens is outlined and illustrated by diagrams.

Diagrammatic illustrations are provided of the various types of apparatus used, including a plan of the cabinet for demonstrating clearly the smaller embryos mounted between watch glasses. Photographic examples of the results achieved are also shown.

INTRODUCTION

The writers of this article, after working for many months with the alizarin red S method of demonstrating bone formation in embryos from the different domestic animals and in 1-6 day old mice, feel that their results may well be of use in many laboratories, particularly if a "short cut" is provided by these working instructions. Many articles leave much experimenting to be done by the interested technician before perfection is obtained; this may not always be possible owing to pressure of routine work and other factors beyond his control.

The methods and observations are designed principally to reduce time wastage to a minimum, while giving the best results. It is hoped to continue this work, using teleostean fishes and other forms of aquatic life; these results will be published at a later date.

This method is a direct descendent from the technic of Schultz (1897) and the alizarin red S modifications of Lipman (1935), and Cumley, Crow and Griffen (1939). These were tried with varying degrees of success, the main objection being a delay in getting the final result, all important, when such work has to be done by a tech-

nician already inundated with his routine work. Cumley, Crow and Griffen's method (1939) requires a minimum of 10 to 14 days according to size, compared with 7-11 days. This saving in time has been achieved with no loss in quality. (See photographs).

METHOD

The embryo to be used should preferably be fresh and without the formation of hairs, or (in the case of chicks) feathers. Should it be found necessary to use specimens which have already formed feathers, they ought to be plucked thoroughly; clipping should be avoided as the roots remain opaque, giving rise to objectionable spots when photographed.

An incision should be made in the abdomen and the viscera carefully removed from thorax and abdomen. The prepared specimen should now be treated as follows:

1. Place in 95% alcohol for three days.
2. Place in 2% potassium hydroxide until the skeletal structure shows through the musculature (approximately 3 days, though longer may be required for larger specimens, taking care that there is no disintegration). (Formalin fixed specimens have been used with fair results, but require an infinitely longer time in this bath, and in some cases it was increased in strength to 5% potassium hydroxide.)
3. Transfer without washing to a 1:10,000 aqueous solution of alizarin red S and stain for 6-12 hours.—The embryo will now be a reddish purple. If understained, it should be returned to the stain.
4. Transfer specimen to 2% potassium hydroxide for 1 day.
5. Transfer specimen to Solution No. 1 (differentiator) and place in an incubator at 70°F. for 2 days.

Solution No. 1: 2% potassium hydroxide,
0.2% formalin,
Glycerin (pure)

Equal parts of the three solutions.

6. Transfer to Solution No. 2 and place in incubator at 70°F. for 1 day.

Solution No. 2: 2% potassium hydroxide 100 ml.
glycerin (pure) 400 ml.

7. Transfer to final bath (pure glycerin) and place in incubator at 70°F. until properly cleared, approximately 1-2 days.

When carrying the specimen through the various stages, it is advisable to have it in a glass receptacle so that the degree of staining

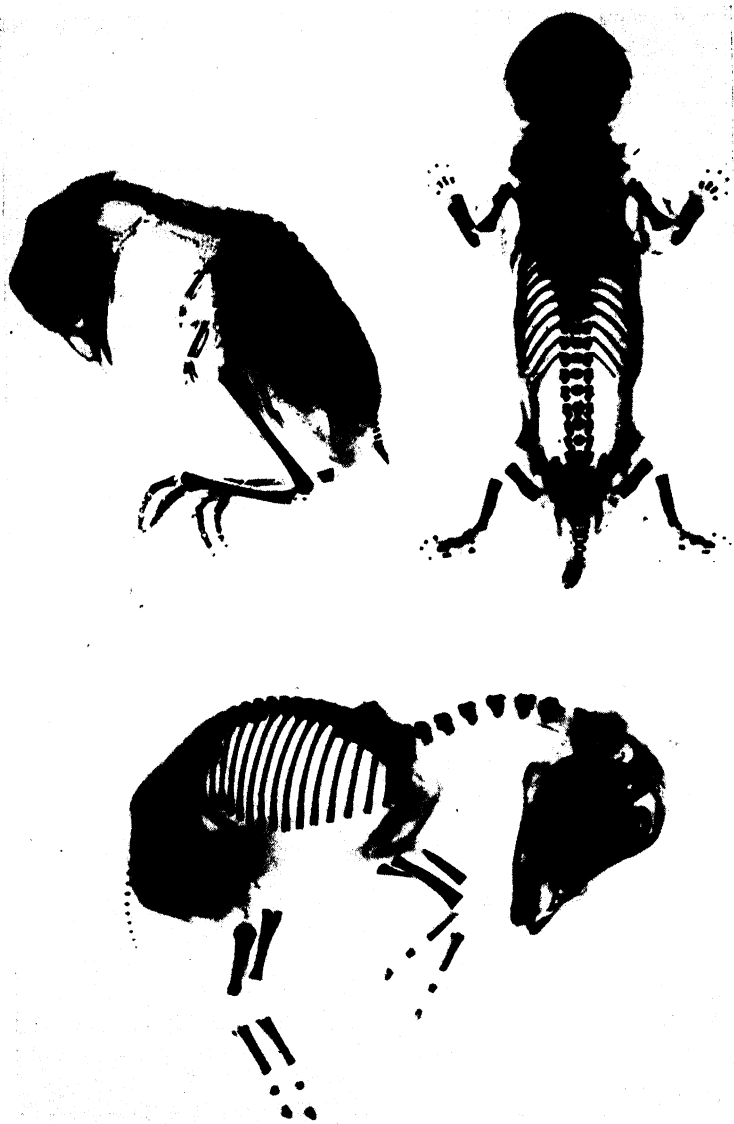


PLATE 1.

Top, left: Newly hatched chick. Right: 2 day old mouse
Bottom: Foal, approximately 4 months.

or differentiation can be seen at a glance by simply holding the container over a lamp.

Methyl benzoate has been used for clearing as supplementary to the method just mentioned; beautifully clear specimens were obtained, but the amount of shrinkage was so great as to render them unsuitable where it was necessary to retain, as nearly as possible, the natural shape. The specimen should now appear translucent with the skeletal structure showing red. If the muscular tissue retains some stain, the specimen ought to be further treated with acid alcohol, 1% sulfuric acid and 95% alcohol, until the desired effect is obtained. Since this solution attacks the tissues and bone it ought to be controlled in use. It has been found by the writers that it is advisable to leave the musculature faintly stained and so show the skeletal relationship.

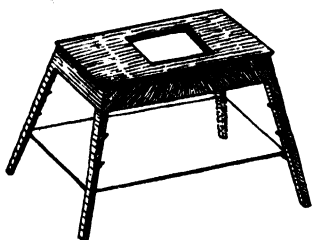


FIG. 1.—Stand for photographing unmounted large specimens.

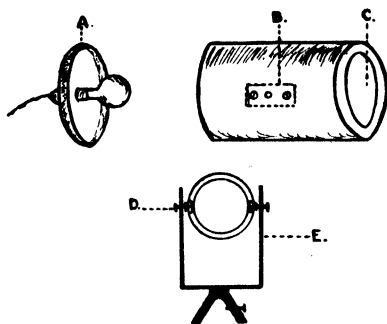


FIG. 2.—Lamp House for Photoflood.

- A. Lid with bulb holder.
- B. Wood screwed to side of tin.
- C. Opening cut in bottom.
- D. Screws for holding in position.
- E. Slots for raising and lowering.

Bright light tends to weaken the color in time and should be avoided. Wherever overstaining is apparent and undesirable and it is feared that further reduction with the aid of acid alcohol is likely to be detrimental to the specimen, an improvement can be made by placing the dish containing the specimen in strong sunlight for periods to be determined by the amount of reduction required. Many writers suggest the use of ultra violet rays. This method has not been tried and cannot be commented upon.

The final glycerin should be free from even the most minute foreign bodies, and ought to be filtered.

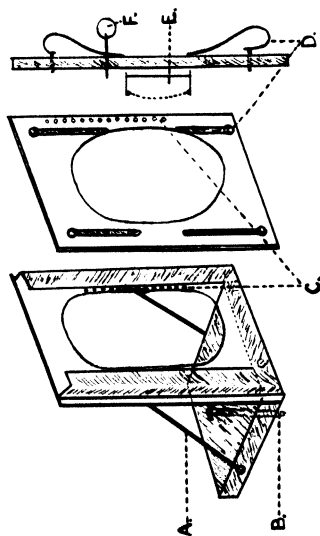


FIG. 3.—Adjustable stand for holding watch glass mounted specimens on Micro Bench.

- A. Supporting stays.
- B. Screw for fixing to bench.
- C. Holes for altering height.
- D. Spring clips.
- E. Condenser.
- F. Pin for locking.

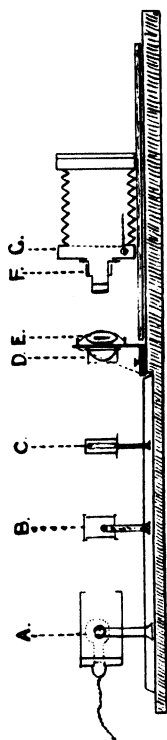


FIG. 4.—Arrangement for photographing embryos between watch glasses.

- A. Photoflood bulb.
- B. Condenser.
- C. Green filter with tissue paper.
- D. 3" macro condenser.
- E. Embryo between glasses.
- F. 3.25 Aldis lens on sliding flange.
- G. Camera front with sleeve.

After transferring the stained specimen to the final glycerin, it will be necessary to see that all air bubbles are removed from the thorax, abdomen and mouth and that none remain to be objectionable after mounting. Placing the dish containing the specimen in an oven or incubator at approximately 70°F. for 2 hours will dislodge all air bubbles adhering to the sides of the dish, the specimen itself and in the glycerin, but cannot be relied upon to remove those trapped in the thoracic or abdominal cavities. To remove these, a camel's hair brush should be used; by tilting the specimen the bubbles can be encouraged to float to the surface.

Avoid using forceps for fear of damaging the fragile bones. Use a scoop for transferring and a glass rod for maneuvering.

PHOTOGRAPHING THE SPECIMEN

No elaborate photographic equipment is necessary to get excellent results. Indeed, the equipment used to procure the results shown at the end of the article consisted of a quarter plate Thornton Pickard Reflex camera with 4.5 Ross Xpres lens of 6-inch focal length, and an F 6 Aldis lens of 3.25 focal length (R.M.S. fitting) which can be screwed into the nosepiece of a microscope, a home-made stand for absolute rigidity, Fig. 1, a spot lamp, Fig. 2, and adjustable slide platform with a Watson 3-inch Macro condenser fitted, Fig. 3, and an Ilford Junior Floodlight or a Kodak standette, with a 275 watt type S Photoflood. (See figures for constructional details of the stands mentioned.)

Starting with the smaller embryos, that is, anything up to 30 mm., it is more satisfactory to have them mounted between watch glasses before photographing. It generally happens that the larger specimens will be photographed before the final mounting: this method of mounting will be described later in the section dealing with mounting. A standard photoflood bulb fitted into the spot light described is placed on the microbench and alignment adjusted (See Fig. 4).

This gives very satisfactory results, provided a diffusing screen is placed in the filter holder along with the filter; this is necessary to obtain even lighting, since the watch glasses, filled with glycerin, create what amounts to a bi-convex lens.

Ground glass and flashed opal were tried, but the best results were obtained by the use of a piece of ordinary white tissue paper. This had the desired effect and an evenly illuminated image resulted.

The watch glasses with the embryo sandwiched between them are placed on the adjustable slide stage and so arranged that the condenser is evenly covered. The stage is screwed in position on the microbench and the 3.25 Aldis lens fitted to the lens panel of the

camera by means of a felt-lined flange with suitable screw (Fig. 4).

One focuses on the object and, if necessary, extends the bellows to get a greater magnification, sliding the flange with the lens to and fro to obtain a crisp image. Owing to the depth of the subject, it is imperative that the diaphragm be closed to about F22, in order that all parts of the skeleton will be as sharp as possible. As conditions

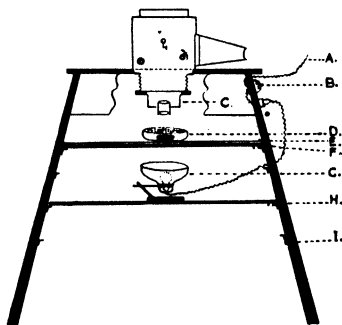


FIG. 5.—General arrangement for photographing larger specimens.

- A. Lead to switch controlling light.
- B. Switch.
- C. Lens box.
- D. Glass vessel containing specimen.
- E. Tissue paper or thin white paper.
- F. Plate glass.
- G. Floodlight.
- H. Plywood shelf.
- I. Brass brackets.

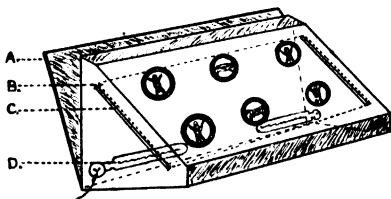


FIG. 6.—Demonstration Cabinet.

- A. Hinged back.
- B. Diffusing screen.
- C. Supports for screen.
- D. Tubular bulbs.

vary, it is unnecessary to give the exposures here, except as a very rough guide:

Plate—Ilford Rapid Process Pan.

Diaphragm—F 22.

Filter—Wratten B, Green.

Exposure—3 seconds.

Ilford Standard M.Q. Developer—3½ minutes.

Temperature—70°F.

For all embryos larger than 30 mm. the T.P. Reflex was used with the lens box reversed to give a longer extension and consequently a larger image.

The specimen is placed in a suitable glass receptacle, at the required distance from the lens on top of a sheet of plate glass covered with thin white paper (2 sheets of tissue paper works equally well).

An Ilford Junior Floodlight with standard Photoflood is placed underneath, shining through the glass, paper and specimen, and arranged for even lighting. An orange filter is used behind the lens for this method.

Since the lighting is static, it will be found that once the diaphragm is decided upon, preferably about F 22, the exposures will generally be of the same duration.

The general lay-out for this type of photograph is shown in Fig. 5.

Ilford Rapid Process Pan. Plates were used, and were developed in I.D2 for $3\frac{1}{2}$ minutes at 70°F.

MOUNTING AND DEMONSTRATING

Little information seems to have been published about this very important subject. Nowhere could we find practical suggestions which were up to the standard necessary for the modern museum or collection.

These tried methods and suggestions are recorded for the benefit of the newly interested who may not have attempted them, and they may also form a working comparison for those already expert.

Again the size of the embryo governs the method, and considerable time was spent before a satisfactory end was reached.

MOUNTING BETWEEN WATCH GLASSES

This method is essentially for the smaller embryo (anything up to 25 or 30 mm.) and has a great deal in its favor by virtue of the fact that it assures a compact and easily demonstrated specimen, beautifully clear, with a distinct saving in glycerin. Two 3-inch watch glasses are prepared by grinding the edges with fine carborundum powder which has been sprinkled on a sheet of plate glass. Optimum evenness should be aimed at, making sure that the ground surfaces meet closely. Care should be taken to avoid scratching the viewing surfaces.

The embryo which is ready for mounting should be placed in a fairly large vessel containing glycerin which has been boiled to free it from any air bubbles. The specimen should never be placed in the glycerin until perfectly cool. Then the temperature should be further reduced to approximately 40°F. by placing the container in the refrigerator or cold chamber. Should the latter not be available and the cooling process be omitted, greater care will have to be exercised during the following stages to prevent the inclusion of air bubbles between the glasses.

To mount the specimen between watch glasses successfully without air bubbles, it was necessary to adopt the following method.

Place the first of the watch glasses in the cooled glycerin under the embryo and arrange the position. The second glass should be slid into the glycerin at an angle in such a manner that, when it comes into contact with the second one, it will expel any air carried down by it. Once the ground edges are in contact, the watch glasses are squeezed together tightly, all glycerin cleaned away from the outside, and then dried. This must be done thoroughly or the sealing mixture may not adhere properly.

A thick seal should be built all round, and leveled with a hot spatula. Once set, a coat of shellac should be applied and allowed to dry. A hard yet resilient sealing medium was required and the following made up and used with success:—

Bitumen asphalt.....	3 pt.
Canada balsam.....	1 pt.
Paraffin wax 48°C.....	2 pt.
Pitch.....	1 pt.

The medium mentioned in a later paragraph for fixing the glass rods can also be used, but should it ever be necessary to re-open the watch glasses, it is doubtful if this could be managed without breaking them.

For larger specimens, clock glasses have been used with effect where the specimen could be held in position by the narrowing sides of the glass, and in cases where the specimen has no great depth, the use of one clock glass, ground as before, and cemented to a piece of good quality glass has proved invaluable.

MOUNTING IN JARS

The largest specimens can be mounted in polished front specimen jars of suitable dimensions, first cutting a panel of glass the required size and drilling a hole at the top large enough to admit a piece of glass rod tightly, as long as the width of the jar. This prevents the panel from tipping back or forward. (One should never use tubing, if possible, as the rod is less visible and makes a neater job once completed). Next, one drills two or more holes, depending on the size of the specimen, into which glass rods equal to approximately half the width of the jar are inserted. The ends are heated to soften and pressed down on a suitable block to cause a slight swelling. They are then cemented into place with the following mixture:—

Litharge (finely powdered).....	3 drachm.
Fine white sand.....	3 drachm.
Plaster of Paris.....	3 drachm.
Resin (powdered).....	1 drachm.

These are mixed together with *Ol. lini*, to which a little dryer has been added and allowed to stand for about four hours. It must then be

used within the next four hours. A thin coating is applied to the cleaned glass. The rods are so arranged that the embryo can be partially supported, since it is soft, while the main support is provided by means of the finest quality catgut, anchored by the sealing medium. It will then be almost invisible once in the glycerin.

DEMONSTRATION CABINET

The diagram (Fig. 6) herewith shows the type of cabinet used by the writers for demonstrating embryos mounted between watch glasses which can be made quickly and inexpensively. The diffusing screen consists of two sheets of plain glass with three sheets of tissue paper between them, placed about $1\frac{1}{2}$ inches away from the specimen. The light is provided by two 3-inch tubular type bulbs fitted to holders fixed to the ends, so that they lie horizontally along the back of the cabinet at the greatest distance from the screen. The cable is led to the outside of the case and a switch provided, allowing the light to be turned off or on at will, so that no appreciable heat is allowed to generate within the case and any possible ill-effects from prolonged lighting are reduced to a minimum. The low wattage bulbs used (25 W.) have been left burning for periods up to two hours during tests and have been found satisfactory.

SUMMARY

1. The alizarin red S method of staining is specific for developing and ossified bone.
2. An appreciable saving of time has been achieved over other methods referred to in the text.
3. Photographic, mounting and demonstrating methods are given, with diagrams.
4. Photographs of embryonic horse, chick and 2-day old mouse are shown.

ACKNOWLEDGMENT

The authors wish to thank Professor T. Grahame, Anatomy Department, Royal (Dick) Veterinary College, for his encouragement and advice.

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NOTES ON TECHNIC

PROTARGOL: OLD AND NEW

Stocks of protargol of foreign origin are becoming exhausted, and since such protargol is no longer available, the American-made product must be used for histologic staining. Some of the earlier domestic protargol seems not to have given satisfaction in neurohistology; therefore it seemed desirable to compare the staining qualities of the pre-war material with several lots furnished recently by Winthrop-Stearns, Inc., New York City.

The tests were made on two samples of cat tissue: (1) cerebellum; (2) a carotid artery specimen which included vagus nerve branches and a small autonomic ganglion of the region of the carotid sinus. These tissues were selected because they stained particularly well when old protargol (Lot No. N-051) was used, and also because they contained various types of nerve cells and a range of nerve fiber sizes from very fine to coarse. The tissues were fixed 2 days in: picric acid (saturated aqueous), 85 ml.; formalin, 15 ml.; and trichloroacetic acid, 0.5 g. Paraffin sections 10μ thick were stained by the fast-green-protargol technic (Stain Tech., 22, 49-50, 1947) and by a modification thereof in which staining was done at 60°C. for only 2 hr. Fresh solutions were used for each test.

The following lots of protargol were compared: N-051 (foreign) and A-29, 182-CB and 335-CB (domestic). The slides were processed in groups of four, one for each sample of protargol, in order to eliminate causes of variations other than those caused by the protargol itself.

When staining was done at 37°C. for 40-48 hr., all samples of protargol stained well, and with such uniformity that it was not possible to tell with certainty one slide from another. At 37°C., and after 20-24 hr. in the staining solutions, the old protargol (N-051) stained more strongly than the new. At 60°C. for 2 hr., staining by new protargol was not satisfactory, in that the small caliber nerve fibers were not stained, and the other neural elements were pale. The old sample stained satisfactorily.

These tests suggest that, insofar as neurological staining is concerned, the new, domestic protargol behaves as if it were more stable (or less reactive) than the old. If, however, the tissue possesses good staining qualities and sufficient time be allowed for staining, the new protargol is capable of giving a type and quality of stain indistinguishable from the old. Although no tests were made with

tissue that did not stain well even with old protargol, it is a safe guess that such tissue would be refractory toward new protargol.

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Medical School, Chicago, Ill.*

AN IMPROVED METHOD FOR ATTACHING PAPER LABELS TO SLIDES

If 70% ethyl alcohol is substituted for water, paper labels (gummed with mucilage) can be securely attached to slides with minimum effort by the following procedure:

After the mounting medium on the slides has dried, clean the end where the label is to be placed thoroughly by means of a cloth moistened with xylene. Allow the xylene to evaporate, or if you wish to proceed immediately, clean the slide again by means of a cloth moistened with alcohol. Pipette a few drops of 70% ethyl alcohol onto the space where the label will be placed. Do not moisten the label with water, but simply place it directly on the alcohol. Press it firmly with a clean towel. The alcohol will moisten the label and a firm attachment will result.

With this method, label varnish can be added sooner than usual since the alcohol evaporates more rapidly than water. Also, it can be noticed that fewer (if any) air islands are left between the label and slide, as is often the case when water is used.

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LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

LARIONOV, L. P., and BRUMBERG, E. M. **Living and dead cells under the ultraviolet microscope.** *Amer. Rev. Soviet Med.*, 5, 111-14. 1948. *From Comptes Rendus (Doklady) Acad. Sci. URSS. n. s.*, 54, 264-70. 1946.

The writers employ the ultraviolet microscope of Brumberg and Hershgorin, modified by use of an achromatic objective (aperture 0.5). Such a microscope has been used by previous workers mainly on fixed tissue; but the writers follow Brumberg's work on living cells at a wave length of 260 m μ , which reveals a nuclear structure identical with that observed in fixed preparations. The equipment employed by the present authors is such that focusing can be done in visible light, thus avoiding exposure of the living cells to the ultraviolet rays until the first photograph is made. Living cells were photographed in tissue cultures prepared with the hanging drop method on a quartz coverslip; the coverslip with the culture was placed upon a quartz slide in a drop of Ringer's solution. The writers find that nuclear elements (except nucleoli) of intact living cells show no absorption of ultraviolet rays of wave lengths from 254 to 275 m μ ; but when the cell is damaged, ultraviolet rays begin to be absorbed. The cytoplasm of a killed or damaged cell loses its ability to absorb ultraviolet rays. The writers conclude that this technic offers new possibilities for the study of living cells. Photomicrographs are included in the paper to illustrate the points discussed.—H. J. Conn.

DYES AND THEIR BIOLOGICAL USES

SNYDER, H. R., and MEISEL, S. L. **The synthesis of azo boronic acids. II. Dyes from tetrazotized benzidine-2, 2'-diboronic acid.** *J. Amer. Chem. Soc.*, 70, 774-6. 1948.

The possible development of an irradiation therapy based on the nuclear disintegration process occurring when the boron isotope of atomic weight 10 captures a slow neutron has provoked an interest in the synthesis of boron-containing dyes having structures similar to those of Trypan blue and Evans blue. Such substances might be expected to concentrate in certain tissues, which might then be irradiated with slow neutrons with the result that the energetic nuclear disintegration would be localized. Accordingly, analogs of Trypan blue and Evans blue have been prepared by the coupling of tetrazotized benzidine-2, 2'-diboronic acid with 1-amino-8-naphthol-3, 6-disulfonic acid and with 1-amino-naphthol-5, 7-disulfonic acid, respectively.—R. T. Whittenberger.

ANIMAL MICROTECHNIC

BARNICOT, N. A. **The supravital staining of osteoclasts with neutral-red: their distribution on the parietal bone of normal growing mice, and a comparison with the mutants grey-lethal and hydrocephalus-3.** *Proc. Roy. Soc. London B*, 134, 467-85. 1947.

Animals were examined at birth, 7, 10, 14, 18, 22, and 28 days. For supravital staining they were killed with ether, the skin stripped from the head, and the skull roof removed with scissors under a Ringer drip. Pieces were transferred to a 1:10,000 solution of neutral red (Gurr) in Ringer and incubated at 37°C. for $\frac{3}{4}$ to 1 hour. Before examination the dura was removed by carefully stripping it forward, starting from the tentorium cerebelli.

For quantitative studies and studies with abnormal animals the parietal bone was used. The whole skull was transferred from the stain to 2% HgCl₂. The

dura was removed 2 to 3 hours later. The osteoclasts remained sharply stained in this solution for several weeks. Fixed preparations are not satisfactory.—*T. E. Weier.*

COPELAND, D. EUGENE. The cytological basis of chloride transfer in the gills of *Fundulus heteroclitus*. *J. Morph.*, 82, 201-27. 1948.

Studies of the chloride cells in the gills of *Fundulus* were made under varying experimental conditions, by the use of reagents capable of indicating location and relative amounts of chloride and mitochondrial material. Chloride test used was a modification of the Leschke method: (1) fix 18 hours in AgNO_3 , 1 g., HNO_3 (concentrated) 2 ml., distilled water, 98 ml; (2) rinse $\frac{1}{2}$ hour in several changes of distilled water; (3) develop 5 to 10 hours in D-11 (Eastman Kodak formula) diluted by addition of 3 parts distilled water; (4) rinse briefly and treat 2 or 3 hours in acid fixing bath F-5 (Eastman Kodak formula) diluted by addition of 4 parts distilled water; (5) wash in running tap water or repeated changes of distilled water for 1 or 2 hours; (6) dehydrate, embed in paraffin. Use absolute darkness for tissues in AgNO_3 - HNO_3 solution, indirect light for fluid changes up to treatment with hypo; after which ordinary light may be used. A schedule for gold toning, to blacken the brownish shades is also given. The user is cautioned that these methods do not give good cytological fixation.

Mitochondrial preparations are made as follows: (1) fix 24 hours in Regaud (3% potassium dichromate, 4 parts, neutral formalin, 1 part); (2) rinse about $\frac{1}{2}$ hour in several changes of distilled water; (3) postchromate in 3% potassium dichromate for 3 days at room temperature; (4) rinse as in 2, dehydrate, embed in paraffin, and section at 4 or 5 μ ; (5) stain with Altman's acid fuchsin; (6) differentiate in saturated picric acid in absolute alcohol, 5 parts, 20% alcohol, 95 parts. Both fixation and postchromation must be done in a dark-box.—*Elbert C. Cole.*

HOGEBOM, GEORGE H., SCHNEIDER, WALTER C., and PALLADE, GEORGE E. The isolation of morphologically intact mitochondria from rat liver. *Proc. Soc. Exp. Biol. and Med.*, 65, 320-1. 1947.

Rat liver was homogenized in the all-glass apparatus of Potter and Elvehjem (*J. Biol. Chem.*, 114, 495, 1936.) with 0.88 *M* sucrose to give a final concentration of 1 g. of tissue in 10 ml. of homogenate. Nuclei were removed by centrifuging three times at 600 \times gravity for 10 minutes. Centrifugation of the supernatant for 20 min. at 24,000 \times gravity resulted in the sedimentation of mitochondria and microsomes. Resedimentation at 24,000 \times gravity resulted in the separation of the mitochondria with the microsomes remaining in the supernatant. The mitochondria so prepared retained their rod-like shape and stained with Janus green in a dye concentration of 1/20,000. They were stable in 0.88 *M* sucrose for several days at 4° C., and were found to contain 70 to 80% of the succinoxidase activity of the original liver homogenate. Authors emphasize that isotonic saline is unsuited to the morphological preservation of mitochondria and suggest that the intracellular osmotic pressure at the mitochondrial membrane may be considerably higher than the blood osmotic pressure.—*H. A. Davenport.*

LEWIS, MARGARET REED, and GOLAND, PHILIP P. In vivo staining and retardation of tumors in mice by acridine compounds. *Amer. J. Med. Sci.*, 215, 282-9. 1948.

Oral administration of 331 acridine compounds (26 non-amino, 270 mono-amino and 35 diamino) was made to tumor-bearing mice, in known percentages of dry weight of their food. (A table has been prepared showing the structural formulae of the 331 acridine compounds studied, their source, percentage used, number of days fed, size of tumor and color at autopsy of the treated mice. The compounds are arranged as substitutes of acridine based on the number of amino groups present on the acridine structure. Access to this table or a microfilm of it may be had at the Wistar Institute.) Of the group 204 stained tumor tissue (0 non-amino, 187 monoamino and 17 diamino). The majority of compounds that colored tumors also brought about some retardation in their growth. The amount of retardation caused by 33 of the acridine compounds resulted in tumors that were 1/10 to 1/18 the size of those in untreated mice and 16 of them

retarded tumor growth to such an extent that when the untreated mice died (14 to 16 days) with tumors measuring 8,000 to 11,000 cu. mm., the mice that had been fed these compounds were healthy and had tumors measuring 200 to 455 cu. mm. or 1/20 to 1/40 that size. The administration of acridine compounds did not prevent tumor growth or bring about regression of growing tumors. It simply slowed the rate of multiplication of tumor cells. The majority of the 9-amino acridines that stain tumor tissue as well as retard its growth show dialkyl-amino-alkyl-amino chains in the 9-position. Analysis of the results in acridine dyestuffs and antimalarials has shown the importance of the presence of amino groups as substitutes of the acridine structure for staining.—*J. A. Kennedy.*

MANWELL, REGINALD D., and FEIGELSON, PHILIP. A modified method of preparing the J. S. B. stain. *J. Lab. & Clin. Med.*, 33, 777-82. 1948.

Several modifications of the original method (*J. Lab. & Clin. Med.*, 30, 1078-82, 1945) are given. They involve filtering out the precipitate resulting from the acid oxidation of methylene blue and redissolving it in M/20 Na_2HPO_4 , which serves both as a solvent and a buffer. Spectrophotometric tests show that the active staining agent is a mixture of azure B and methylene blue in a proportion of about 3 to 1. A solution of commercial azure B and medicinal methylene blue in these proportions was found to be a good substitute for the laboratory-prepared product, although apparently not quite equal to it.—*John T. Meyers.*

MOHS, FREDERIC E. The preparation of frozen sections in the chemosurgical technique for the microscopically controlled excision of cancer. *J. Lab. & Clin. Med.*, 33, 392-6. 1948.

The microscopic control of excision attained by the chemosurgical treatment of cancer is dependent upon the completeness of frozen sections which have been fixed *in situ* prior to excision. The following is recommended: Mount the section in a small pool of albumin which has been partially frozen. Flatten any irregular specimen with spatula handle, or elevate any depressed edges. Orient so that the knife strikes the narrowest part of the specimen first. When frozen, cut sections about 15 to 25 μ thick, and place in a glass dish of cold water containing a few drops of a surface tension depressant such as Zephiran to prevent disruption of fragile tissue. Place on slide and stain as follows: Put in 95% alcohol for 15 seconds or more, dip quickly in thin Parlodion and put in Delafield's hematoxylin for 2 minutes or longer if necessary. Dip in acid alcohol (25 ml. of concentrated HCl made to 500 ml. with 70% alcohol), put in water with a little NaHCO_3 until blue, dip in eosin (Put 2.5 g. aqueous eosin in 500 ml. of water, add 4 ml. of concentrated HCl and 1 ml. of glacial acetic acid. Pour off the supernatant liquid and wash the precipitate 6 times with water. Filter, dry, and dissolve in 100 ml. of 95% alcohol). Treat 2 minutes with equal parts of alcohol and ether, then absolute alcohol to remove Parlodion, clear in carbol xylene, pass through xylene and mount in Clarite.—*John T. Meyers.*

MICROÖRGANISMS

BOIVIN, A., TULASNE, R., and VENDRELY, R. Localisation et rôle des deux acides nucléiques chez les bactéries: le problème du noyau bactérien. *Compt. Rend. Acad. Sci.*, 225, 703-4. 1947.

Ribonucleic acid and deoxyribonucleic acid are located in the bacterial cell with the aid of the enzymes ribonuclease and deoxyribonuclease, followed by Giemsa stain. Bacteria of the enteric group have been used as material. Details of the method are not given. Ribonucleic acid is peripheral in the bacterial cell and most abundant in young cultures. Deoxyribonucleic acid is confined to a small dense nucleus.—*T. E. Weier.*

SAXER, E. Beobachtungen über die Säurefestigkeit der Tuberkelbazillen. *Schweiz. Zts. Path. Bakt.*, 10, 388-402. 1947.

When 10% HCl is employed for 1 hour, it does not destroy the virulence of tubercle bacilli and does not interfere with their demonstration in animal experi-

ment or in cultivation. This method is to be favored in contrast to the employment of 25% H_2SO_4 . The use of the HCl solution for 3 hours seems to produce a favorable effect on the growth of the bacteria on culture media.—*F. Polgar*. (Courtesy *Biological Abstracts*).

HISTOCHEMISTRY

BONDY, PHILIP K., and SHELDON, WALTER H. **Histochemical demonstration of liver glycogen in human diabetic acidosis by liver biopsy.** *Proc. Soc. Exp. Biol. and Med.*, **65**, 68–70. 1947.

A core of liver tissue was secured by the Silverman needle at varying times during the treatment of patients in diabetic acidosis. The low glycogen content before treatment and restoration after treatment is illustrated by three photomicrographs of the tissue stained by Gomori's method (G. Gomori, *Amer. J. Clin. Path.*, **10**, 177, 1946.). Authors report that a quantitative correlation between histochemical and chemical methods for the determination of liver glycogen is being studied, and that the outlook for a satisfactory means of correlation is favorable.—*H. A. Davenport*.

FISHER, ISADORE, and GLICK, DAVID. **Histochemistry XIX. Localization of alkaline phosphatase in normal and pathological human skin.** *Proc. Soc. Biol. and Med.*, **66**, 14–18. 1947.

In searching for phosphatases and lipase in human skin, results were negative for acid phosphatase and lipase. Alkaline phosphatase activity was observed in the stratum granulosum, endothelial lining of capillaries, in hairs, hair follicles and sweat glands. Pathologic tissue from cases of lupus erythematosus, psoriasis and papular urticaria showed no significant changes in enzyme activity. Proliferating fibroblasts, scar tissue and infiltrate of pyogenic granulomas reacted positively for the alkaline phosphatase. In chronic exema and acne vulgaris, the perivascular infiltrate was positive.—*H. A. Davenport*.

GOMORI, G. **Histochemical differentiation between esterases.** *Proc. Soc. Exp. Biol. and Med.*, **67**, 4–6. 1948.

Organs were fixed in cold acetone, dehydrated and embedded in paraffin. Many organs were placed in the same block to facilitate ease of comparisons of enzyme activity. The effects of cholate, quinine, arsanilate, eserine, pilocarpine, urethane, caprylate, n-butyraldehyde, acetophenone, hexylresorcinol, and NaCl were studied. Author concludes, "Differences in the behavior of esterases (lipases) from various sources toward activator and inhibitor substances, similar to those found previously in *in vitro* experiments, can be observed also in tissue sections stained for lipase."—*H. A. Davenport*.

LEBLOND, C. P., PERCIVAL, W. L., and GROSS, J. **Autographic localization of radio-iodine in stained sections of thyroid gland by coating with photographic emulsion.** *Proc. Soc. Exp. Biol. and Med.*, **67**, 74–6. 1948.

The thyroids of animals treated with radioactive iodine were fixed in Bouin's fluid or formalin, embedded in paraffin, sectioned 3 to 5 μ , stained, dehydrated, coated with 1% celloidin and dried 6 hr. Coating with the photographic emulsion was done in a darkroom at 2 or 3 feet from a No. 1 Wratten safelight. The emulsion of Kodak medium lantern slide plates was softened by soaking in distilled water for 10 min. at $19^\circ \pm 1^\circ \text{C}$. The soft emulsion was scraped into a beaker and heated to 38–39° C. just before coating. The stained slides were warmed to 38° C. and 4 to 8 drops of emulsion applied over the sections of tissue and smoothed quickly with a camel's hair brush. Tilting the slide from side to side effected an even coating and gelation was allowed to occur with the slide resting on a level surface. The slides were stored in light-tight containers at 0–2° C. and kept dry with P_2O_5 . Slides were developed at intervals (length of interval not given) to determine the proper exposure. Development was 2 min. in Kodak D-72 at $19^\circ \pm 1^\circ \text{C}$. and fixation 10 min. in acid fixer. Slides were washed 20 min. at a temperature below 20° C. Dehydration and covering in balsam completed the process. Slides should be dried at room temperature. Short exposures tend to give better cytological localization of radioactivity. Preparations can be studied with high power magnification.—*H. A. Davenport*.

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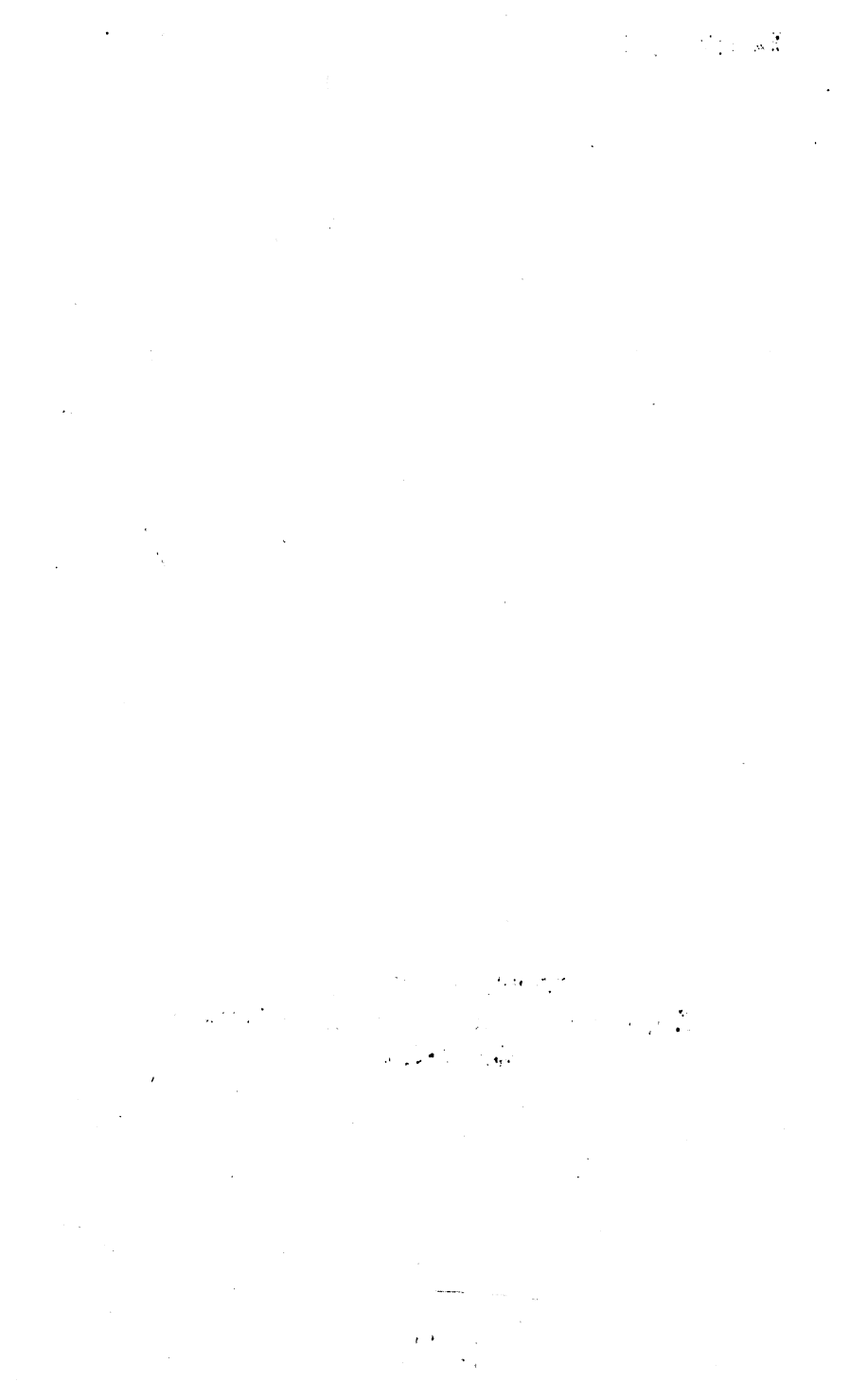
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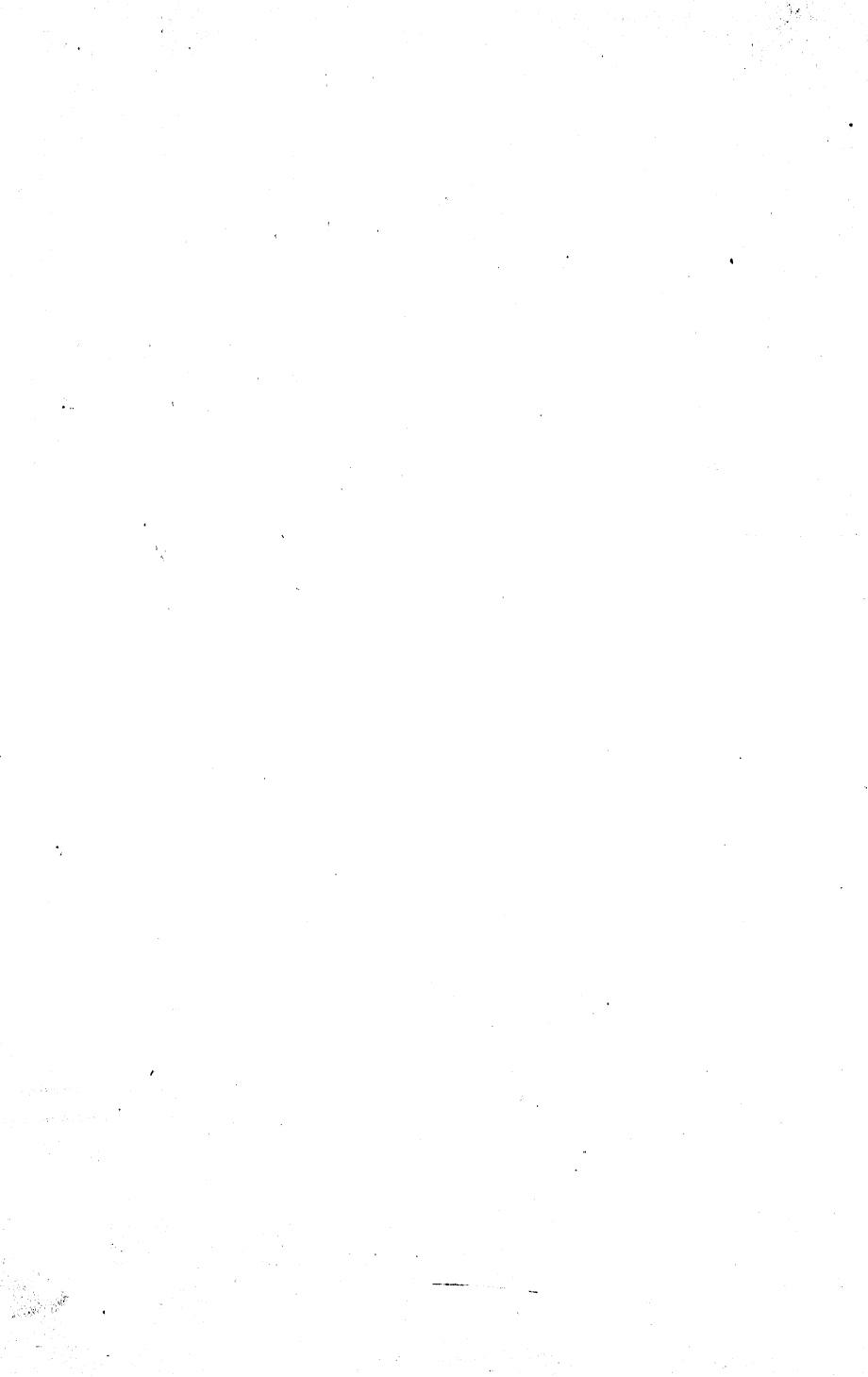
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A COMPARISON OF AMERICAN STAINS WITH RECENT GERMAN PRODUCTS¹

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ABSTRACT.—In 1947, 31 samples of stains of German manufacture were collected in that country, with the coöperation of the U. S. Department of Commerce and one of the American stain manufacturers. They have been compared with current American products by the same tests as used for the samples submitted for certification. Only two proved distinctly superior to the corresponding American dyes, three others as good as the best American samples, four proved entirely unsatisfactory, while seven others were abnormal from the spectrophotometric standpoint although performing satisfactorily when used in the staining procedures by which they were tested.

During and shortly after World War I, American biologists were faced with a shortage of the accepted German stains or with American stains of questionable suitability. In the period between the wars, however, American dye manufacturers, in close coöperation with a group of biologists especially interested in staining and known as the Biological Stain Commission, have developed standardized stains of high quality. Opportunity to learn how favorably these American stains compare with those of recent German manufacture was lacking until 1947 when a series of 31 German biological stains were collected² in Germany and made available for study by the Biological Stain

¹This work was supported in part by the National Aniline Division, Allied Chemical and Dye Corporation; Coleman and Bell Co., Inc.; and Hartman-Leddon Company.

²These samples were collected by Mr. J. G. Kern, Mr. I. Thornton, and Mr. B. M. Helfaer at the direction of Mr. J. D. Nantz of the National Aniline Division, and distributed by the Office of Technical Services of the U. S. Department of Commerce.

Commission. These stains were subjected to chemical, spectrophotometric, and staining procedures similar to those regularly employed in testing stains submitted by American manufacturers. The results of these tests in comparison with typical American stains form the basis of this communication.

EXPERIMENTAL

The color characteristics of the stains were studied using the Beckman DU Quartz Spectrophotometer at suitable concentrations as determined by current studies on the use of this instrument for routine spectroscopic examination. With each stain, the position and height of the absorption curve were recorded, as well as the mathematical ratio of the color densities at two selected wavelength points on either side of the absorption maximum. These points are generally taken at $+15$ and -15 $m\mu$ of the characteristic maximum, and the ratio of color densities at these points is an expression of the symmetry of the absorption curve near the peak. Since definite points are selected for comparison, e.g. D520/D550 for a stain with a characteristic peak at 535 $m\mu$, a sample of this stain having an abnormal position of the absorption maximum is also certain to have an abnormal ratio. Hence this ratio is of secondary importance in comparison with the position of the absorption maximum, but has nevertheless been found of use in the optical characterization of stains. The height of the absorption peak at a definite concentration of stain in standard cells (i.e. the absorption coefficient) has been used in this study as a measure of the dye content or strength of the stain, although the validity of this measure has not yet been established with each of the stains.

The staining properties of the dyes have usually been tested either in our laboratories or in collaborating laboratories³ by the procedures published in Conn's Biological Stains, 5th Edition, pp. 275-310, although for some of the stains additional tests were made to give a better check on their performance.

The following table includes the names of the German stains submitted, the apparent American equivalent, and a comparison of the spectrophotometric data and staining properties of these dyes with other pertinent notes. In examining the data it should be kept in mind that the ranges reported for American samples are

³Credit is extended to the following collaborators for their part in these investigations: F. B. Adamstone, E. T. Bell, Sylvia H. Bensley, A. B. Dawson, C. A. Doan, S. I. Kornhauser, Lillian M. Leavitt, R. D. Lillie, B. C. Nesin, O. W. Richards, Sara A. Scudder.

representative but not inclusive. Thus individual samples in a given series of American stains may have been unsatisfactory, although these would not have appeared on the market. Neither can a single sample of a German stain be considered typical; it might be better or worse than the average. (The ranges given for American stains are incidentally not necessarily those indicating approval by the Biological Stain Commission since more extensive investigation is in progress in these laboratories.)

CONCLUSIONS

Of the 31 German stains tested, 30 could be directly compared with typical American stains. Of these, 20 were essentially similar in staining and spectrophotometric tests to their American analogs. Three of the German stains, namely *toluidinblau*, *indigokarmin*, and *safranin* were reported as "excellent"; while *azokarmin G* and *B* were found superior to the usual American stain encountered. On the other hand, four of the German stains were considered unacceptable for their staining uses: namely, *rosanilin*, *fuchsin*, *phloxin*, and *pyronin*. The last three of these were also abnormal from the spectrophotometric standpoint. In addition to these, *baumwollblau*, *wasserblau*, *fuchsin*, *Congorubin*, *rose Bengal*, *Sudan III* and *gentianviolett* were also abnormal in their color characteristics, but since these were reported satisfactory for staining, some might have been approved if submitted as American samples. Further details are recorded in the Table.

As a general conclusion, it seems evident that the German stains, as represented by the 31 samples tested, offer no advantage over American stains. The few German stains reported as excellent or superior are more than counterbalanced by important stains which are quite unsatisfactory in the German series. There remains little or no claim for the superiority of German stains which might have existed during and immediately after World War I.

TABLE 1.—COMPARISON OF SPECTROPHOTOMETRIC AND STAINING PROPERTIES OF AMERICAN AND WORLD WAR II GERMAN STAINS

The samples specified as "M.C. No. —" are the German samples.

Those not so designated represent composite data on typical American samples.

German Name and American Equivalent	Absorption Peak (M μ)	Ratio of Color Densities*	Color Density† (D _s)	Staining Quality, Acceptability and Remarks
Methylenblau—"stand" M.C. No. 236	665 (water)	D650/D680 = 1.44	193	Typical sample, acceptable.
Methylene blue	663-664	D650/D680 = 1.40-1.60	193-233	
Toluidinblau—"stand" M.C. No. 217	637 (water)	D610/D640 = 0.86	88.0	Excellent staining results. Typical of late American samples. Satisfactory chemically.
Toluidine blue O	625-637	D610/D640 = 0.90-1.10	94.0-110	
Nilblau—"stand" M.C. No. 235	644 (50% alcohol)	D620/D650 = 0.81	152	(a) Satisfactory staining. (b) Fair but passable staining. Satisfactory chemically.
Nile blue	637-655	D620/D650 = 0.67-1.22	86.0-186	
Baumwollblau M.C. No. 230	613 (water)	D590/D620 = 0.86	51.0	Staining satisfactory. Decolorization test required more time and alkali. Absorption peak and ratio considerably different from American samples. Not acceptable as "Aniline Blue".
Aniline blue W.S.	592-605	D590/D620 = 1.08-1.20	40.0-67.0	
Wasserblau—"stand" M.C. No. 211	613 (water)	D590/D620 = 0.86	44.0	Staining satisfactory. Weaker than American aniline blues or methyl blues (C.I. No. 706). Decolorization test satisfactory. Absorption peak and ratio atypical. Not acceptable as "Aniline blue".
Aniline blue	592-605	D590/D620 = 1.08-1.20	40.0-67.0	

Kristallviolet-"stand" M.C. No. 227	590 (water)	D575/D605 = 1.04	230	Satisfactory, staining and chemically.
Crystal violet	590	D575/D605 = 1.11-1.12	210-245	
Methylviolet-"stand" M.C. No. 231	582 (50% alcohol)	D575/D605 = 1.50	195	Staining satisfactory. More red than American samples. Acceptable.
Methyl violet	585-587	D575/D605 = 1.33-1.51	200-225	
Gentian violet M.C. No. 233	585 (water)	D575/D605 = 1.48	115	Staining satisfactory. Low color intensity. Not acceptable chemically.
Gentian (Methyl) Violet**	586	D575/D605 = 1.38-1.41	195-215	
Indigokarmin-"stand" M.C. No. 219	610 (water)	D595/D625 = 1.02	36.0	Excellent staining results. Satisfactory chemically.
Indigo carmine	608-610	D595/D625 = 1.03-1.06	37.3-44.0	
Azokarmin B-"stand" M.C. No. 203	511 (water)	D500/D530 = 0.96	23.0	"Azokarmin B" similar in spectrum to G, lower color intensity, but excellent stain. "Azokarmin G" showed strong and brilliant staining, resistant to phosphotungstic acid, stronger than American stains in color. Very satisfactory.
Azokarmin G-"stand" M.C. No. 218	512	D500/D530 = 0.99	30.0	
Azocarmine G	512	D500/D530 = 0.98-1.00	24.5-25.5	Only passable as connective tissue stain. Satisfactory as Andrade indicator. Excellent and precise staining in Bensley's technic. Satisfactory chemically.
Saurefuchsin M.C. No. 215	544 (Dilute acid)	D530/D560 = 1.25	64.0	
Acid fuchsin	542	D530/D560 = 1.37-1.58	73.0-87.0	

TABLE 1—Continued

German Name and American Equivalent	Absorption Peak ($M\mu$)	Ratio of Color Densities*	Color Density† (D_s)	Staining Quality, Acceptability and Remarks
Fuchsin M.C. No. 223	552 (50% alcohol)	$D_{530}/D_{560} = 0.82$	173	"Fuchsin" satisfactory for T.B., Endo, and Bacteriostatic; unsatisfactory for Feulgen. Not acceptable as either rosanilin or pararosanilin chemically. "Rosanilin-stand" completely unsatisfactory for T.B. staining and Verhoeff's technic. Very poor for Feulgen, Endo, and Bacteriostatic. Typical rosanilin chemically.
Rosanilin- "stand" M.C. No. 206	549 (50% alcohol)	$D_{530}/D_{560} = 0.99$	267	
Rosanilin	548-549	$D_{530}/D_{560} = 0.90-0.99$	247-260	
Pararosanilin	545-546	$D_{530}/D_{560} = 1.16-1.27$	253-263	
Congorubin- "stand" M.C. No. 208	520 (water)	$D_{490}/D_{520} = 0.79$	34.0	Staining only fair. Color more like acid fuchsin; does not contrast well with iron hematoxylin. Spectroscopically much redder than American samples. Would not be accepted.
Congo red	498-499	$D_{490}/D_{520} = 1.11-1.12$	43.0-63.0	
Erythrosin- "stand" M.C. No. 191	526 (water)	$D_{510}/D_{540} = 0.84$	90.0	Staining satisfactory. Spectrum shows a more purple constituent not typical of American samples.
Erythrosin	526-527	$D_{510}/D_{540} = 1.02-1.20$	86.0-100	
Neutralrot- "stand" M.C. No. 213	542 (acid 50% alcohol)	$D_{525}/D_{555} = 0.99$	118	Staining satisfactory. Most satisfactory for supravital. Not toxic to cells nor any impairment. Typical chemically, high color intensity.
Neutral red	541	$D_{525}/D_{555} = 0.97-1.02$	66.0-114	
Phloxin- "stand" M.C. No. 216	525 (alk. 50% alcohol)	$D_{530}/D_{560} = 3.32$	76.0	Unsatisfactory staining—deep red, too intense. Spectroscopically too yellow, not typical of American samples. Not acceptable.
Phloxin	545-547	$D_{530}/D_{560} = 1.06-1.36$	88.0-140	

Safranin-"stand" M.C. No. 209	532 (50% alcohol)	D515/D545 = 1.01	116	Excellent staining, especially for Flemming triple stain. Chemically typical and acceptable.
Safranin	532-533	D515/D545 = 1.00	130-140	
Pyronin-"stand" M.C. No. 192	545 (50% alcohol)	D530/D560 = 1.35	42.0	Staining fair on G.C. smear, not bright red. Unsatisfactory in Scudder technic; too weak, did not penetrate. Spectroscopically more yellow than Pyronin Y. Weak in color intensity. Would not be accepted.
Pyronin B	544-555	D530/D560 = 0.48-0.60	56.0-118	
Pyronin Y (G)	547-550	D530/D560 = 0.80-1.00	60.0-176	
Bordeaux B-"stand"† M.C. No. 204	—	—	—	Staining satisfactory.
Orange G-"stand" M.C. No. 232	480 (water)	D470/D500 = 1.04	34.6	Staining satisfactory. Chemically typical of American samples.
Orange G	476-479	D470/D500 = 1.01-1.06	28.0-39.3	
Eosin-"stand" M.C. No. 224	516 (dilute alkali)	D500/D530 = 1.25	104	Staining satisfactory. Chemically acceptable.
Eosin Y	517	D500/D530 = 0.96-1.10	104-120	
Lichtgrün-"stand" M.C. No. 225	631 (water)	D620/D650 = 1.21	59.0	Staining satisfactory. Chemically acceptable.
Light green	632-633	D620/D650 = 1.18-1.37	70.0-100	
Malachitgrün-"stand" M.C. No. 193	617.5 (water)	D605/D635 = 1.22	207	Staining satisfactory. Typical chemically.
Malachite green	617.5	D605/D635 = 1.22-1.28	180-200	
Brilliantgrün-"stand" M.C. No. 205	630 (50% alcohol)	D615/D645 = 1.02	217	Staining only fair, but passable. Chemically typical of of American samples.
Brilliant green	630	D615/D645 = 1.01-1.03	203-223	

TABLE 1—Continued

German Name and American Equivalent	Absorption Peak ($m\mu$)	Ratio of Color Densities*	Color Density† (D_s)	Staining Quality, Acceptability and Remarks
Auramin M.C. No. 234	431 (water)	D420/D450 = 1.24	124	Satisfactory staining. Dissolves slowly, needs filtering, slightly oxidized. Spectroscopically typical of American samples.
Auramine O	431	D420/D450 = 1.22-1.27	130-140	
Alizarinrot S-"stand" M.C. No. 229	555, 595 (dilute alkali)	D545/D575 = 1.11	34.0	Satisfactory staining. Typical chemically.
Alizarin red S	556, 595	D545/D575 = 1.09-1.12	35.3-40.6	
Bengalrose-"stand" M.C. No. 220	551 (alk. 50% alcohol)	D540/D570 = 3.32	64.0	Satisfactory staining. Considerably different spectroscopically from American samples.
Rose bengal	553-558	D540/D570 = 1.10-2.08	70.0-92.0	
Methylorange-"stand" M.C. No. 228	508 (dilute acid)	D490/D520 = 0.92	114	Satisfactory staining. Essentially same spectroscopically as American samples.
Methyl orange	508	D490/D520 = 0.90-0.91	126-130	
Sudanrot III-"stand" M.C. No. 188	515 (benzene)	D490/D520 = 0.84	75.0	Satisfactory staining. Visually and spectroscopically more like American Sudan IV samples.
Sudan III	510	D490/D520 = 0.89-0.90	71.2-83.7	

*See text.

$$\dagger D_s = \frac{\log I_0/I}{c \times l}$$

"specific density", where I_0 and I are the intensities of light emerging from the solvent and sample cells respectively, c is the concentration of the dye sample in g. per liter, and l is the length of the light path in centimeters.

**This series refers to samples submitted under the name "Gentian violet" by one of the American manufacturers.

†Not submitted by American manufacturers.

POLLEN TUBE CULTURE ON A LACTOSE MEDIUM

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ABSTRACT.—A modified sugar-agar technic for growing pollen tubes is described in which a Van Tieghem cell is used as a moist chamber, and 12% lactose substituted for the normally used sucrose. The advantages offered over previous technics are: (1) direct observation of germination and growth at any stage and for any period of time, (2) easier prevention of bacterial contamination with the long growth periods required to obtain pollen tube divisions, (3) simple method for making permanent slides which are suitable for cytological study.

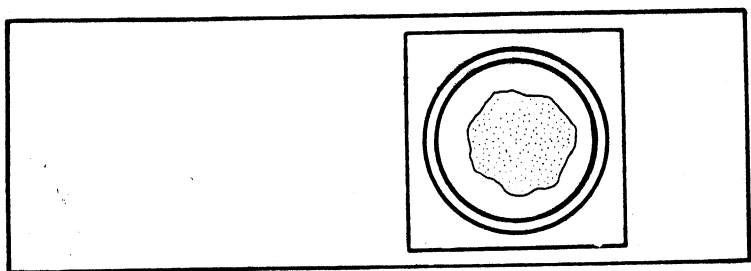
During recent years increased use of pollen tubes in the study of cell sensitivity to physiological changes and to the effects of short wave radiation has made it necessary to develop a technic for growing the tubes which is simple, has the least chances of failure, and will allow proper staining and easily made permanent slides that are suitable for cytological study.

Most technics employed at the present time make use of slides coated with a layer of sucrose agar, dusted with pollen, and placed in a moist chamber. The method reported in this paper is a modification of this technic which has certain advantages over that in general usage.

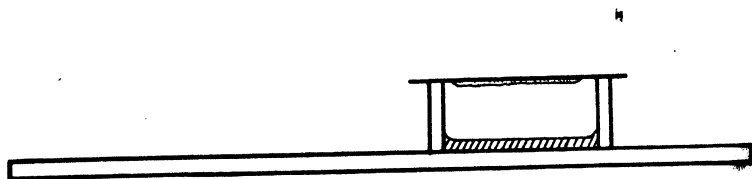
The agar medium is spread on a 22 mm. square cover glass instead of a slide, and the moist chamber is provided in the form of a Van Tieghem cell constructed from a glass ring attached to the slide by means of a small amount of vaseline or Canada balsam, and containing several drops of water in the bottom (Fig. 1). The cover glass with agar medium is inverted over the glass ring, using a little vaseline to seal it. In a very short time the air within the Van Tieghem cell becomes saturated, and thus prevents the agar from drying.

The second modification is that lactose is used in place of sucrose as the type of sugar to prepare the medium. As lactose has the same empirical formula as sucrose, and hence the same molecular weight, equal amounts of either sugar give a solution with the same osmotic pressure. The medium used for the growth of pollen of *Tradescantia paludosa* contained 3% agar, 12% lactose, and 0.01% colchicine made up in 25 ml. lots with distilled water.

The relatively high concentration of agar is used because it is easier to spread on the cover glass in a thin even coat. The medium may be applied with a glass rod or a clean finger-tip. Alternatively, the whole cover glass may be dipped in the hot medium, and then the excess allowed to drain by touching the edge of the cover glass to a piece of paper towelling. When the cover glass is dipped in the solution, a 1% agar medium will give a thinner and more even coat. If this method is used, the agar may be removed from one side of the cover glass after it has solidified.



TOP VIEW



SIDE VIEW

FIG. 1.—Van Tieghem cell adapted for pollen tube culture.

The sugar concentration is higher than that used by most investigators for *Tradescantia* pollen (e.g., see Swanson, 1940), but was chosen after a number of tests because it seemed to give better continued growth, which is essential if the pollen tubes are to develop to the point of the pollen tube division. Lactose was chosen in place of sucrose for two reasons: (1) preliminary tests seemed to indicate that it gave results which were slightly better than with sucrose, and (2) it was much simpler to prevent bacterial contamination with the lactose medium.

The colchicine was added to the solution to inhibit the development of the spindle mechanism. This prolongs the metaphase

period, and thus causes metaphase figures to increase in number on the slide. It also allows the chromosomes to spread out in the long, narrow pollen tube. Ideally, they are strung out one after the other (Fig. 2), though in actual practise this is relatively infrequent.

In preparing cultures with the pollen from *Tradescantia paludosa* the following technic was used. Freshly opened flowers were picked in the early morning, and the pollen dusted on the medium directly from the anthers with the aid of a camel's hair brush. Improved germination and growth were found when the lactose-agar was spread very thinly, an effect also noted by Beck and Joly (1941). This necessitated a minimum time between spreading the agar and completion of the culture in order that the medium remain moist—a maximum of about two minutes.

The thinness of the agar medium used in this method allows the pollen tubes to grow in only one plane. This is of considerable



FIG. 2.—Colchicine-inhibited metaphase of the pollen tube division in *Tradescantia*.

importance in the analysis of slides, particularly if photography is required.

Germination percentages of 80% to 95% were normally obtained with this method. On the medium used, the time required for the pollen to reach the metaphase stage of the pollen tube division was in some cases as low as eight to ten hours, but generally was a minimum of about twelve hours. In one lot of 27 cultures, an average of 43 countable metaphases per cover glass was obtained, with the maximum at 121 metaphases.

When the pollen tubes have been growing for 13–15 hours, the cultures may be fixed and permanent slides made in the following manner. The cover glass is removed from the Van Tieghem cell, placed in alcohol-acetic (3:1) for a few seconds, and then inverted on a large drop of acetocarmine on a slide. The preparation may be examined at this point and any failure eliminated. After staining for several minutes, the cover glass is removed with a pair of tweezers, passed through the following dehydration series:

- (1) 3 parts 70% alcohol, 1 part glacial acetic acid.
- (2) 3 parts absolute alcohol, 1 part glacial acetic acid.
- (3) 9 parts absolute alcohol, 1 part glacial acetic acid.
- (4) absolute alcohol.

and mounted in diaphane. A prolonged immersion (more than 15 seconds) in absolute alcohol, causes the development of crystals in the medium and makes analysis of the slide difficult or even impossible.

DISCUSSION

One important advantage of this technic is that the growing pollen may readily be observed¹ for any length of time without danger of the culture drying up or the growth being injured in any way. Eigsti (1940) found that pollen tube growth may begin within 15 minutes after sowing. Though varying with the kind of pollen and the concentration of sugar used, with this technic it can be seen that in 10-12% lactose the grains frequently begin to form pollen tubes between two and three minutes after sowing, have mostly germinated in five minutes, and any that do not germinate in fifteen minutes probably never will develop. The method is ideal for class demonstration of cell growth and cytoplasmic streaming. With optimum conditions pollen tubes have grown 500 μ in an hour, and cytoplasmic streaming was measured at 200 μ a minute.

Active streaming in some tubes may continue for as long as 36 hours, but normally when the tubes reach their maximum length, in *Tradescantia* usually 2000 to 3000 μ (though it may be 5000 or more if the tube is very narrow), the end becomes enlarged and frequently bursts, spewing out the nuclear contents.

It is rather interesting that lactose, which is a non-nutrient sugar as far as pollen grains are concerned, can be used with results as good as with the nutrient sugar, sucrose. This shows that for pollen tube growth, which in terms of the size of the original pollen grain is tremendous, external nutrient is not required. Apparently the function of the sugar is solely as a non-toxic substance which regulates the external osmotic pressure to the point where water may be absorbed at a slow rate, so that growth may be achieved without pollen tube bursting.

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¹With some microscopes, better lighting is obtained by removing the top lens of the condenser, thus increasing its focal length.

THE SELECTIVE DEMONSTRATION OF RED BLOOD PIGMENTS (HEMOGLOBIN AND ERYTHRO- CRUORIN) IN MICROSCOPIC BALSAM PREPARATIONS¹

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ABSTRACT.—Using smears of Amphibian testis, after fixation with a 5% aqueous solution of sulfosalicylic acid, it was found that the cytoplasm of erythrocytes can be stained selectively by means of a 0.1% aqueous solution of acid fuchsin. Subsequent experiments with whole mounts of *Tubifex rivulorum* proved that this very selective and simple stain for hemoglobin can also be used for the selective demonstration of erythrocrucorin in invertebrates.

Up to now there has been only one method that makes possible the selective demonstration in microscopic balsam preparations of both hemoglobin in vertebrates and erythrocrucorin (Svedberg and Eriksson, 1933) in invertebrates, both in the cellular and the dissolved state. This is the method of Lepehne (1919) which depends on the reaction of the red blood pigments with benzidine, a reaction which is also obtained with peroxidases under different experimental conditions. After Prenant (1921) had shown that the method of Lepehne can also be applied to the "hemoglobins" of invertebrates, the method was modified by Slonimski (1927), Slonimski and Lapinski (1932) and Doherty, Suh and Alexander (1938) and its utility thereby considerably enhanced. The method described in this paper has nothing in common with the basic principle and the modifications of Lepehne's reaction, although it is just as selective, and applicable to the same objects.

The method was discovered in the following way: smears of Amphibian testis were fixed in aqueous sulfosalicylic acid solution, run through a series of descending alcohols to distilled water and stained with iron hematoxylin (Heidenhain). A very selective nuclear stain is obtained after this fixation, but since I also wanted to observe the cytoplasm of the cells, the smears were dipped for about 1 minute into a weak aqueous solution of acid fuchsin. After mounting in Canada balsam, it was noticed that only the cytoplasm of erythrocytes was stained bright red while other cells remained completely unstained.

¹Translated from the German original by Max Alfert, Columbia University.

Control-smears of Amphibian testis, fixed and prepared as before, showed the same selective and intensive staining of erythrocytes, although no mordant and indeed no staining at all preceded the acid fuchsin stain. It became clear that this method constitutes a very selective and simple staining procedure for hemoglobin.

This induced me to determine whether the erythrocrucorin of invertebrates could be demonstrated by the same method, and the small aquatic worm *Tubifex rivulorum* was used for this purpose. This worm, which occurs in large numbers in the mud of brooks, contains the red blood pigment not in blood cells, but like other invertebrates (for instance the larvae of *Chironomus*) in colloidal solution in the blood. The experimental animals were first isolated in small glass containers in clean tap water for 4-5 days; during this time their intestine was emptied of mud which would have partially obscured the course of the blood vessels in the whole preparations required for this purpose. Then the animals were anesthetized by means of ether, dried with blotting paper and put on a glass slide. They were covered with a smaller slide that had been dipped into paraffin and weighted with a small piece of lead, also dipped into paraffin. The covering with a slide only slightly larger than the animal allowed easy access to the fixative, and the paraffin covering made this slide easily removable after alcohol hardening. The weighting of the cover slide flattened the animal so as to permit observations with stronger objectives and the paraffin coating of the weight prevented the lead from dissolving in the fixative. It was found that previous drying with blotting paper removes excess water and causes the object to adhere strongly to the slide. Sulfosalicylic acid being a very strong and rapid protein coagulant causes the object to adhere especially strongly to the slide and diffuses very rapidly—like Carnoy's mixture—through the tissues. But unlike the last named fixing fluid it does not permit any nucleal staining (Feulgen). The narcosis of the animals should not be prolonged and everything should be done rapidly to get them into the fixative with as little alteration as possible.

For this purpose the slide bearing the object is lifted by means of two forceps and put carefully—in horizontal position—into an aqueous solution of sulfosalicylic acid kept ready in a wide pan. The following details of the process are indicated:

- 1) Fix smears and very small animals for 24 hours at 20-25°C. in 5% sulfosalicylic acid in water.

- 2) Transfer whole animals into 2 successive 24-hour baths of 100% * alcohol. Wash smears in 24-hour baths of 100% and 95% alcohol.



PLATE I. EXPLANATION OF FIGURES

Photographs of whole mounts of *Tubifex rivulorum* stained with acid fuchsin, showing selective staining of blood vessels.

FIG. 1 ($\times 50$) FIG. 2 ($\times 250$) FIG. 3 ($\times 520$)

3) Hydrate whole animals in successive 2- to 3-hour baths, smears in 5- to 10-minute baths, of 90, 80, 70, 60, 50% alcohol. Remove the covering upper slide from the whole objects at the beginning of this step, and put slide vertically into the dish.

4) Wash out alcohol in several changes of distilled water over a period of several hours in the case of whole animals, 1 hour for smears.

5) Stain small animals such as *Tubifex*, up to 20 minutes (smears for 1 minute only), in 0.1% aqueous acid fuchsin.

6) Rinse small animals 2 to 3 seconds (smears 1 second), in distilled water. Transfer to 80% alcohol, agitating constantly to quickly remove water, for at most 10 minutes.

7) Treat whole animals and smears in 90% alcohol for 10 minutes or less. Dehydrate smears 20 minutes, small animals 1 to 2 hours in 95% alcohol.

8) Complete dehydration and clearing with 2 changes of 100% alcohol, a 1:1 mixture of 100% alcohol and xylene, and 2 changes of xylene. Mount in balsam.

Fig. 1, 2, and 3 show how selective this method is; the figures show parts of mounted, whole *Tubifex*.

Fig. 1 ($\times 50$) shows the larger vessels dark red (here black) on unstained background in a series of segments.

Fig. 2 ($\times 250$) shows smaller vessels selectively stained in 2 segments.

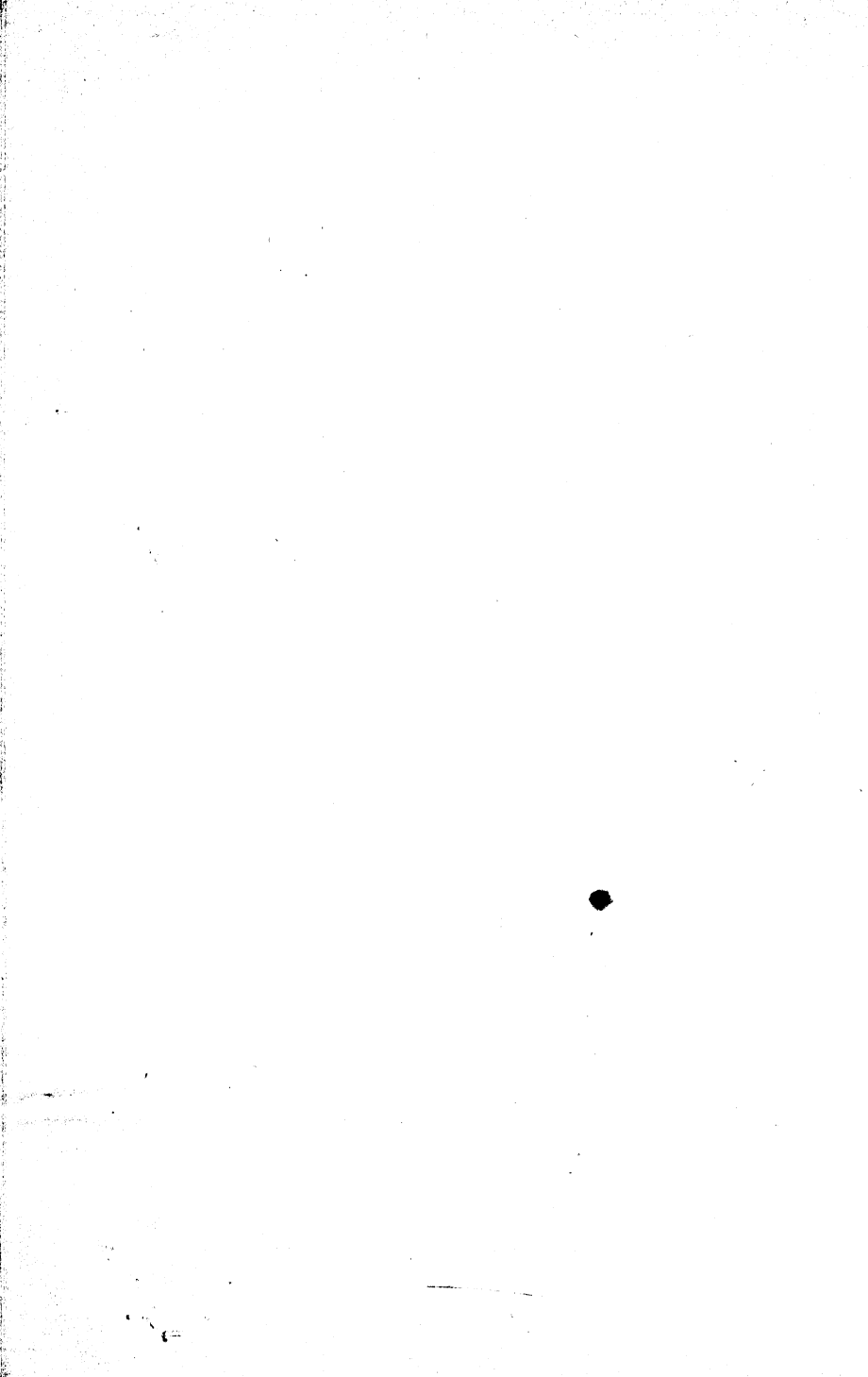
Fig. 3 ($\times 520$, under oil immersion) shows fragments of capillary anastomoses. Although the staining of the vessels is caused only by the selective staining of erythrocrucorin, the illustrations give the impression of injected preparations. The light-grey appearance of other structures, like the intestine in Fig. 1 and 2 or muscle tissue in Fig. 3, is not caused by staining but by a strong reduction of the light which had the purpose of giving considerable depth to the photographs. There is a sole exception to the selectivity of the method, which however is hardly disturbing: in *Tubifex* certain components of the yolk of old oocytes are, interestingly enough, also stained, while all other organs and tissues remain completely unstained. The durability of the preparations is very satisfactory, almost a full year according to my experience.

This paper is based on experiments undertaken at my former Zoological Institute of the University of Lemberg before 1940 and partly on experiments done at the Zoological Institute of the University of Vienna in 1940. For the opportunity to work there I am indebted to Professor Dr. W. Frh. v. Buddenbrock, the former chief of that Institute.

The photographs shown here were taken by Ing. A. Findeis of the C. Reichert Optical Company in Vienna. I thank him for his friendly cooperation.

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A RIGOROUS RE-DEFINITION OF THE PLASMAL REACTION

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ABSTRACT.—The plasmal reaction is here modified so that it is made strictly specific for acetal lipids alone. The two essential points in making such specificity possible are: (1) limiting the duration of the action of HgCl_2 to 2–10 minutes, thus insuring that neither acid hydrolysis nor oxidation participates in the unmasking; (2) obtaining negative controls upon sections of the same block of tissue, these being treated identically in all respects except that immersion in HgCl_2 is omitted. Lipids positive under these conditions are acetals but not necessarily acetal phospholipids. In order to satisfy the second criterion above, material must be either unfixed or fixed for only 1–6 hours in formol. Longer fixation in formol both destroys the acetals and unmasks some non-acetal lipids.

There has been much discussion concerning the histochemical demonstration of carbonyl lipids and fundamentally different opinions have been expressed as to the chemical significance of the methods used. The principal contrasting points of view of American workers are adequately expressed in two recent papers, those of Albert and Leblond (1946) and Wislocki and Wimsatt (1947). Both agree with Gomori (1942) that the plasmal reaction of Feulgen and Voit (1924) is equivalent to the phenylhydrazine reaction of Bennett (1940). They disagree as to whether the lipids visualized are the plasmalogens (acetal phospholipids) of Feulgen and Bersin (1939) or whether they are to an important extent ketosteroids.

The present writer will publish elsewhere the results of an investigation of the significance of the carbonyl lipid reactions. As a necessary preliminary to the study of whether the plasmal reaction and Bennett's reaction are identical, a more rigorously defined plasmal reaction was devised and used. The specificity of this reaction is strictly limited to acetal lipids alone. This paper presents the details of the method.

Carbonyl reagents do not react directly with untreated sections or smears, meaning that tissues contain no free carbonyl compounds in histochemically detectable amounts. Carbonyl groups must first be freed from some masking linkage before they will react. Two

classic examples of this are the nucleal reaction of Feulgen and Rosenbeck (1924), in which short acid hydrolysis releases carbonyl groups from desoxyribose nucleotides, and the Bauer (1933) reaction, in which oxidation makes glycogen reactive to carbonyl reagents. The ultimate reagent used to visualize the unmasked carbonyl compounds has no influence upon the histochemical picture obtained and thus, presumably, none upon the chemical specificity of the reactions. It follows, then, that the specificity of any reaction for carbonyl lipids derives solely from the method used for the unmasking of the reactive products and does not depend upon whether Schiff's reagent, phenylhydrazine or 2, 4-dinitrophenylhydrazine is used.

There are three methods for the unmasking of carbonyl lipids: (1) brief action of HgCl_2 , (2) more prolonged acid hydrolysis (or the action of the acidic Schiff reagent itself), and (3) formol fixation. The first two methods are included in the original definition of the plasmal reaction by Feulgen and Voit (1924). The third method derives from the work of Bennett (1940); Gomori (1942) suggests that the mechanism in this case is oxidative.

The striking action of HgCl_2 upon the acetal bond, splitting it with the formation of a free carbonyl compound, was fundamental to the discovery of the acetal lipids. This lability of plasmalogen toward HgCl_2 enabled Feulgen and Bersin (1939) to recognize that the fatty aldehyde and glycerol of plasmalogen were joined in an acetal linkage. Riesch and Kilpatrick (1935) have described the catalysis of the hydrolysis of the acetal bond by neutral salts, and Meerwein (1927) explains this on the basis of complex formation. Oster and Schlossman (1942) say that HgCl_2 acts "more specifically and quicker" than hydrolysis by mineral acids. It is, of course, necessary to establish the lipid nature of the acetals by extraction of sections in organic solvents.

Acid hydrolysis and formol fixation are less likely to have a specific action upon the acetal bond. Hydrolysis by acid undoubtedly breaks acetal bonds to form free carbonyls but it is quite possible that such treatment may release carbonyls from substances other than acetals. Further, since this process takes an appreciable length of time (1-2 hours), opportunity is offered for other unmasking processes to take place. Oxidation of an acetal bond will split it, but with the formation of an acid rather than a free carbonyl group. Therefore, if the unmasking of lipid substances can be restricted to the action of HgCl_2 alone, it is certain that the reactive lipid originally contained an acetal bond. We cannot, however, be certain that these were phospholipids, for the possibility remains that non-phospholipid acetals exist.

Insistence upon two points in the procedure affords assurance that the unmasking is so restricted: (1) very brief action of HgCl_2 , 2-10 minutes in a 1% solution, to minimize the possibility of the slower actions of acid hydrolysis or oxidation; (2) obtaining negative control sections from the same block of tissue, these sections being treated identically except for the immersion in HgCl_2 . Any material that reacts positively in the control sections cannot be known to be derived from acetal lipids.

The procedure is as follows:

(1) Use unfixed tissues, either frozen sections or smears. Sectioning of such material is aided if blocks are immersed in a dilute gum arabic solution for 5-10 minutes and sectioned in a drop of this solution. Most material can be cut at 15-20 μ by this method.

(2) Wash in several changes of distilled water or physiological saline.

(3) Place one section or group of sections in 1% aqueous HgCl_2 for 2-10 minutes. It seems necessary to allow only sufficient time for penetration to be complete.

(4) Transfer these sections and a control group simultaneously to separate closed dishes of Schiff's reagent. Sections may be left here as long as the controls remain negative but 5-15 minutes is usually sufficient for a maximal reaction to be secured.

(5) Wash both groups of sections in 3 changes of H_2SO_3 , at least 2 minutes in each.

(6) Wash in water; mount in glychrogel (Zwemer, 1933) or in Clarite.

Schiff's reagent or fuchsin-sulfurous acid is preferred as the ultimate demonstrating reagent. Phenylhydrazine (Bennett, 1940) or its 2,4-dinitro derivative (Albert and Leblond, 1946) may be used. With these latter reagents, the preparations are not as distinct, although the results as regards distribution and relative intensity of reacting substances are identical to those with the Schiff reagent. The various ways of preparing the Schiff reagent do not influence the histochemical pictures of the acetal lipids. It is necessary to use a basic fuchsin that is certified for the Feulgen reaction by the Biological Stain Commission, for not all batches of this dye are satisfactory. The best method of preparation is that of deTomasì (1936), using $\text{K}_2\text{S}_2\text{O}_8$. Decolorization is completed with activated charcoal as suggested by Coleman (1938). This results in a colorless solution in which the first hint of returning color can be detected and the solution discarded.

Glass rod section lifters are used to transfer frozen sections. Care must be taken not to allow even traces of Schiff's reagent or HgCl_2

to be carried backward in the series, as this can lead to confusion. Scrupulous rinsing of the lifters after each transfer is advised.

Careful washing in H_2SO_4 is essential to remove all traces of excess reagent that might lead to false staining. This wash solution is conveniently made up as needed by mixing 10 ml. of 10% aqueous NaHSO_4 and 10 ml. of N HCl and diluting to 200 ml. with distilled water.

Sections mounted in glychrogel do not keep. By taking advantage of the fact that the fuchsin-carbonyl lipid compound is practically insoluble in organic solvents, permanent preparations in Clarite may be obtained. The procedure is to float the section upon a slide and to apply successively 50% alcohol, 95% alcohol, absolute alcohol and xylene. This is followed by mounting in Clarite. If a light nuclear counterstain is desired in such preparations, methyl green is quite satisfactory.

Any fixation that neither destroys nor itself unmasks carbonyl lipids could theoretically be used. The only method of fixation known to the author that allows negative control sections to be obtained is brief (1-6 hours) fixation in 10% formol. This makes sectioning much more satisfactory but destroys some of the acetal lipids. Longer fixation in formol will eventually destroy all the acetal lipids as well as unmask positive material in the control sections.

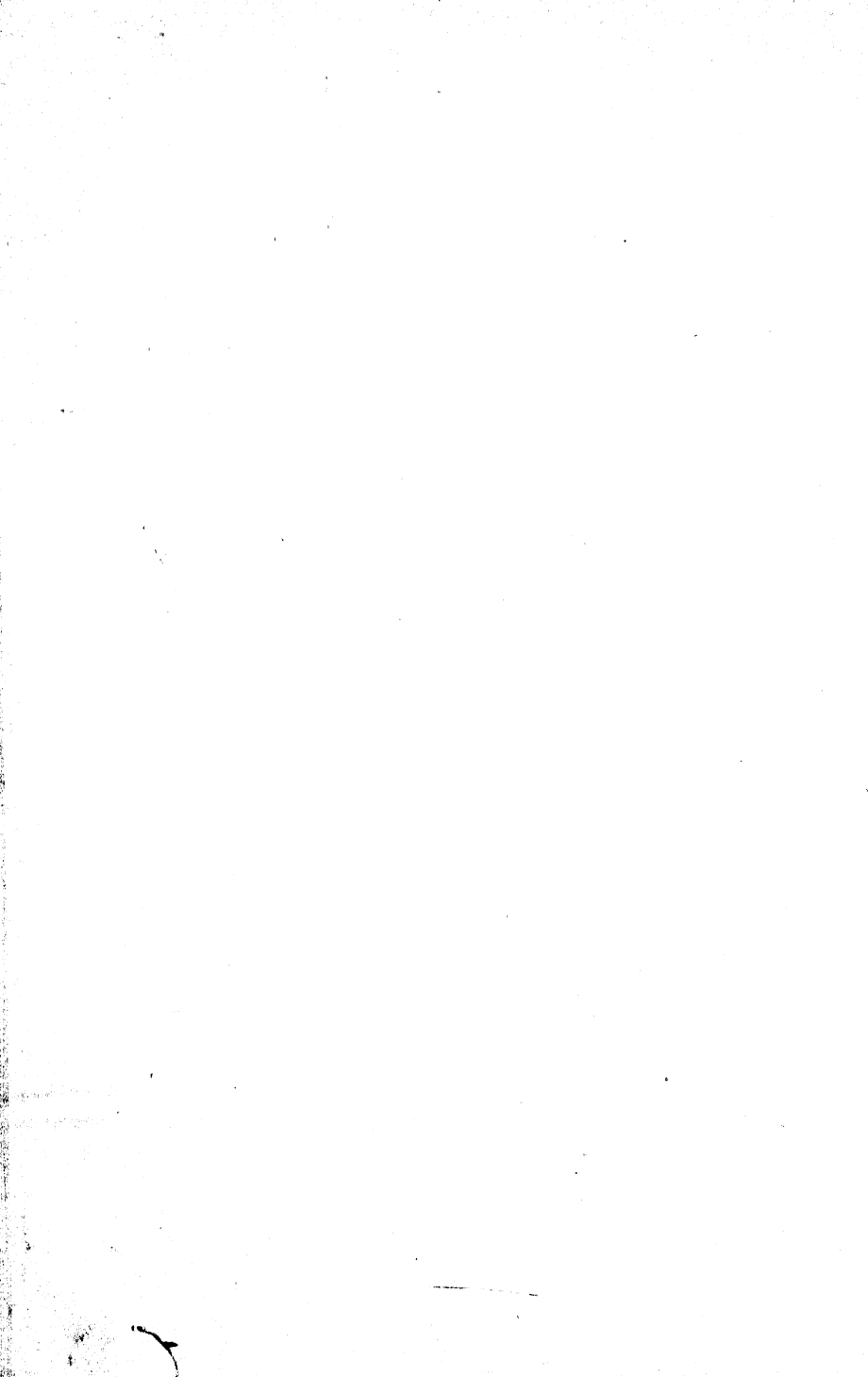
ADDENDUM

Recently it has been brought to the author's attention by Dr. C. P. Leblond and Dr. R. A. Knouff that lipids of the adrenal glands of the mouse and hamster react quickly and directly with the Schiff reagent without any preliminary treatment. Guyon (Comp. Rend. Soc. Biol. 109, 1101-5, 1932) observed that myelin also reacts in this way. Such lipids cannot be proven to be acetals, for the unmasking has not been restricted to the action of HgCl_2 . The carbonyl lipids observed in these isolated cases may exist *in vivo* either actually free or in some bond extremely labile to the acid of the reagent.

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THE HISTOCHEMICAL DEMONSTRATION OF GLYCOGEN BY SILVER COMPLEXES

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ABSTRACT.—Several silver complexes and procedures of hydrolysis for the histochemical demonstration of glycogen were studied. Among these were ammoniacal-silver-hydroxide, methenamine silver, piperazine silver, and ammoniacal silver carbonate. It was found that any combination of acid hydrolysis and oxidation is able to activate the carbohydrate so there is no specific agent although the best was chromic acid. The suggestion is made that periodic acid hydrolysis can yield equally good results in shorter time. Ammoniacal silver carbonate was the best of the complexes studied because it stains rapidly, is economical, stable and easy to prepare.

Direct or indirect (with formol reduction) reactions can be chosen when working with ammoniacal silver carbonate complexes. The following procedures are proposed: a) hydrolysis with 10% chromic acid, 20 to 30 minutes; b) immerse in the following complex: 10% aqueous silver nitrate, 4 ml.; saturated solution of lithium carbonate, to 20 ml.; drop concentrated ammonium hydroxide until almost completely dissolved (leave a light turbidity); complete to 100 ml. with lithium carbonate saturated solution; let stand and filter. Store in a brown glass well-stoppered bottle. The slides can either be exposed 15 minutes at room temperature in this complex and reduced with 2% neutral formol for about 30 seconds, or incubated during 50 to 60 minutes in a 10% solution of said complex at 45–50°C. in the oven followed by a thorough washing with water. Tone with 1:500 gold chloride and finish as usual. Collodion coating and formol alcohol or picro-formol-alcohol-acetic fixation are essential in direct reactions.

So far as we know three methods for the histochemical demonstration of glycogen by silver complexes have been reported (Mitchell and Wislocki, 1944; Gomori, 1946; Arzac, 1947). In all of them two steps are essential: (a) hydrolysis of the polysaccharide; (b) reduction of the silver by the hydrolyzed carbohydrate itself. Except in Gomori's procedure which is a *direct* reaction, the other two need a third step, namely what we may call "development". While two of them (Mitchell and Wislocki, 1944; Arzac, 1947) use potassium perman-

ganate, with bleaching in oxalic acid and AgNO_3 in alcoholic solution, Gomori recommends chromic acid as hydrolyzing agent. For the second step a silver oxide complex of ammonia has been advocated by Mitchell and Wislocki (1944), an ammoniacal silver carbonate by Arzac (1947) and a methenamine complex by Gomori (1946). Since the statement has been made that only methenamine complexes are well suited to be used in direct reactions (in the sense used by Lison, 1936) but we have found ammoniacal complexes extremely sensitive to reduction by glycogen, the present study was undertaken to get information on the possibilities of: (a) replacing or interchanging some steps in both types of histochemical reactions; (b) using other complexes than the ones advocated; and (c) replacing other hydrolyzing procedures than the two previously mentioned.

TABLE 1.—EFFECT OF SEVERAL HYDROLYZING PROCEDURES AND SILVER COMPLEXES FOR THE HISTOCHEMICAL DEMONSTRATION OF GLYCOGEN

Hydrolysis →	A KMnO_4 Oxalic Acid Alc. AgNO_3^*		B KMnO_4 Oxalic Acid Aq. AgNO_3		C KMnO_4 Oxalic Acid Acid AgNO_3^\dagger		D Chromic Acid‡	
	F	H	F	H	F	H	F	H
Complex—↓								
Methenamine-Ag	+	+	—	—	+	+	+	+
Ammoniacal-Ag	+	+	—	—	+	+	+	+
Piperazine-Ag	+	+	—	—	+	+	+	+

*2 to 10% silver solutions (24 hours).

†pH 2 with acetic acid.

‡10% sol. 20–30 minutes.

F=Reduction with formol.

H=Incubation at 45–50°C., or 18–24 hours at room temperature.

B and C=Silver solutions in water.

EXPERIMENTAL

Endometrium, liver, stomach and intestine, spleen, etc. from rodents and human, freshly fixed in alcoholic mixtures, were used. Digestion with saliva (as well as fixation in fluids which do not preserve glycogen) was previously performed when necessary. The different combinations of steps are shown schematically in Table 1. In all cases a double procedure was tried: (a) reduction with formol 0.2 to 4%; (b) acceleration by heat (45°C., no formol), or incubation in the silver complexes during 18–24 hours at room temperature.

RESULTS AND COMMENT

It can be seen in Table 1 that in all cases except B (in which the acidity of alcohol or some acid is lacking) a positive result was obtained in every instance. When formol was used the results were essentially the same for all the combinations of steps and reagents

tried; this was also true when heat was applied, the only essential difference with the latter group being the absence of reticulum. For example, glycogen or mucus plus reticulum (or both) was demonstrated in all the experiments on endometrium, when reduction with 0.25–4% formol was used. When the reaction took place at 45°C. over a period of 30 minutes to 3 hours (18–24 hours at room temperature) reticulum did not appear while glycogen or mucus was demonstrated.

The formulas tried for each complex were varied widely in concentration, solvent and alkalizing agent. It was found that lithium carbonate was the best for ammoniacal and piperazine silver, while ammonium hydroxide for Gomori's was to be preferred. Piperazine works well without any alkalizing agent.

Although the silver concentration of the complex and the speed of the reaction run parallel, from a practical point of view high concentrations are to be preferred when formol is used as reductant since the complexes are quite stable and thus may be used repeatedly.

TABLE 2.—RESULTS OBTAINED WITH SEVERAL SILVER COMPLEXES.

Method		Carbohydrates	Reticulum
Reduction with formol.	Mitchel and Wislocki's	Sharply outlined	Sharply outlined
	Piperazine-silver	Sharply outlined	Not so
	Methenamine-silver	Sharply outlined	Not so
	Ammonium silver-carb.	Sharply outlined	Still less
No reduction	Mitchel and Wislocki's	Blurred	Blurred
	All the others	Well outlined	No reticulum

Glycogen and mucus appear black; grayish purple when scanty and diffuse.

This is particularly true for ammoniacal carbonate complexes. Furthermore silver concentrations as small as 0.004% are sufficient for the demonstration of glycogen in 15 minutes at 45°C. However, sharper images with glycogen and jet black mucus are obtained in 10–15 minutes when 0.25% silver concentrations and formol reduction are used in slides without collodion membrane (see Plate 1). In our experience methenamine silver did not even begin to stain in this length of time at 37–45°C.

Regarding the procedures for hydrolysis, it can be deduced from the results shown in Table 1 that there is no specific hydrolyzing agent for glycogen since appropriate oxidation combined with a more or less independent acid hydrolysis works perfectly. In line with this, Lillie (1948) and McManus (see Wislocki and Dempsey, 1948) have demonstrated that hydrolysis can be performed by periodic acid which fulfills the same requirements, that is acid hydrolysis with

oxidation. Of the methods studied in this work chromic acid hydrolysis was the best. However, it is probable that hydrolysis by periodic acid (not available to us) used with the methods below might give as effective results in shorter time.

In relation to specificity of the technics it can be said definitely that none of the technics so far proposed, either by silver, leucofuchsin or by chromic acid etc., is strictly specific *per se*; all of them demonstrate structures and substances other than glycogen and mucus, so from this particular point of view none of them has advantage over the others. Certain differences, however, can be established from a practical standpoint as shown in Table 2. It can be assumed that progressive reduction of silver by the carbohydrate, while only *adsorption* upon reticulum, takes place in both types of reactions. At high temperatures reduction and blackening of silver by the carbohydrate take place automatically while the latter is carried away from reticulum and other structures with washing. Direct reduction of silver by the carbohydrate is on the contrary too weak when exposed to short periods of incubation or low temperatures so the use of formol is necessary for its visualization. This of course simultaneously reduces and makes visible the silver adsorbed upon the reticulum fibers.

The results of the experiments here reported show, we believe, that because of the many variations that can be made in the ammoniacal silver carbonate complexes (as well as in the technics), the simplicity of their preparation and relatively greater stability and speed for staining, they are preferable. For the investigation of certain tissues like human endometrium, in which variations of reticulum argyrophilia (Fig. 4) during the menstrual cycle represent functional state or activity, an ammoniacal-silver-carbonate complex combined with weak formol reduction is the best choice. The following formula has been demonstrated to be very useful:

"LI2"

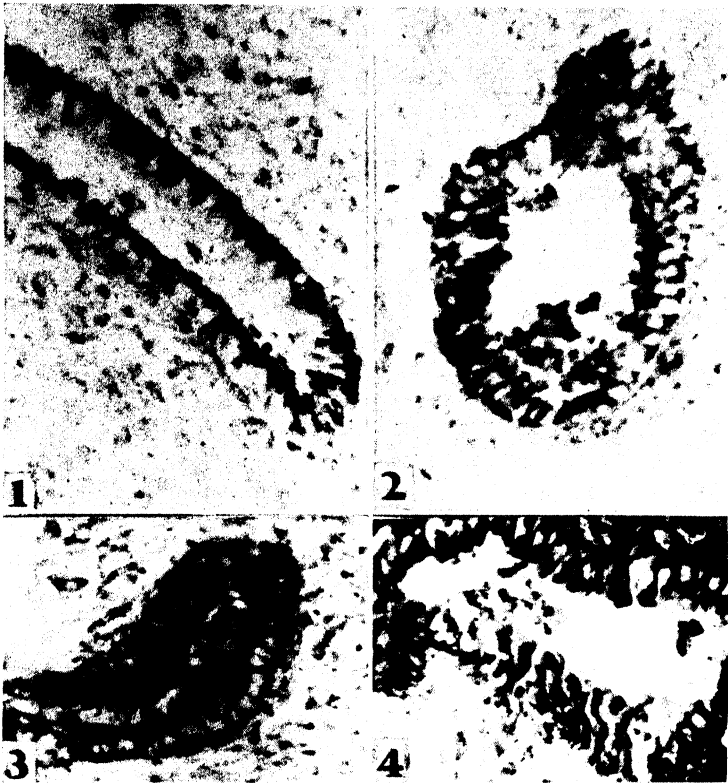
AgNO₃ (10% aqueous solution) 4 ml.
 Li₂CO₃ (saturated aqueous solution) to 20 ml.

Ammonium hydroxide (NH₄OH) drops, until the complex is almost dissolved (leave a very light turbidity)

Li₂CO₃ (saturated aqueous solution) to make 100 ml.

Filter and keep in a dark well-stoppered bottle in refrigerator.¹
 This stock solution can be used repeatedly in full strength (with

¹After 3 months at room temperature this solution has remained unchanged and active.



Human endometrial glands. All from same specimen E. 942 fixed in alcohol-formol. Effect of several silver complexes after 10% chromic acid hydrolysis during 25 minutes.

FIG. 1.—Methenamine silver. Temperature 50°C. Time: 1 hour

FIG. 2.—Piperazine silver (SEP5b). Temperature 50°C. Time: 30 minutes

FIG. 3.—Ammoniacal silver carbonate (LI5b). Temperature 50°C. Time: 30 minutes.

FIG. 4.—Ammoniacal-silver-carbonate (LI2) Room temperature. Time: 15 min. Reduction with 2% neutral formol. Almost no reticulum appears in late progestational phase.

formol reduction) or diluted 1:10 (we call it LI5b) in the oven (without reduction) once or twice. A piperazine silver complex that has worked very well in our hands is the next one:

"SEP1"

Piperazine solution, 0.5%	100 ml.
AgNO ₃ (10% aqueous solution)	1 ml.

This is stock solution. For use dilute 1:4 with distilled water. (SEP5b). Two drops of Li₂CO₃ (saturated aqueous solution) to 50 ml. of SEP5b may be added.

We recommend the following technics: deparafinize and run down the slides to water protecting with collodion² as usual and then:

1. 10% chromic acid solution ——— 20–30 min.
2. Wash thoroughly in tap water and rinse in distilled water.
3. LI2 15 min. at room temp. or in LI5b (10% aqueous solution) or in SEP5b at 45°C. 15–60 min. (Heat the reagent in the oven while hydrolysis is taking place).
4. After cooling wash in water. (In 2% neutral formol 30–60 seconds and then in water if worked at room temperature).
5. Tone with 1:500 gold chloride if desired.
6. Rinse in 3–5% "hypo", 3–5 min.
7. Wash thoroughly. Take off collodion membrane with alcohol-ether.
8. Dehydrate, clear, and mount as usual.

SUMMARY AND CONCLUSIONS

Any kind of pretreatment which involves acid hydrolysis and oxidation permits the histochemical demonstration of glycogen and mucus by silver complexes. Of the procedures that we used chromic acid was the best.

A relatively wide variety of silver complexes are well suited for the histochemical demonstration of glycogen and mucus. Ammoniacal silver carbonate is recommended.

None of the technics so far proposed is specific. Reduction with formol widens the range of non-specificity for all of them but gives sharper images and quicker results.

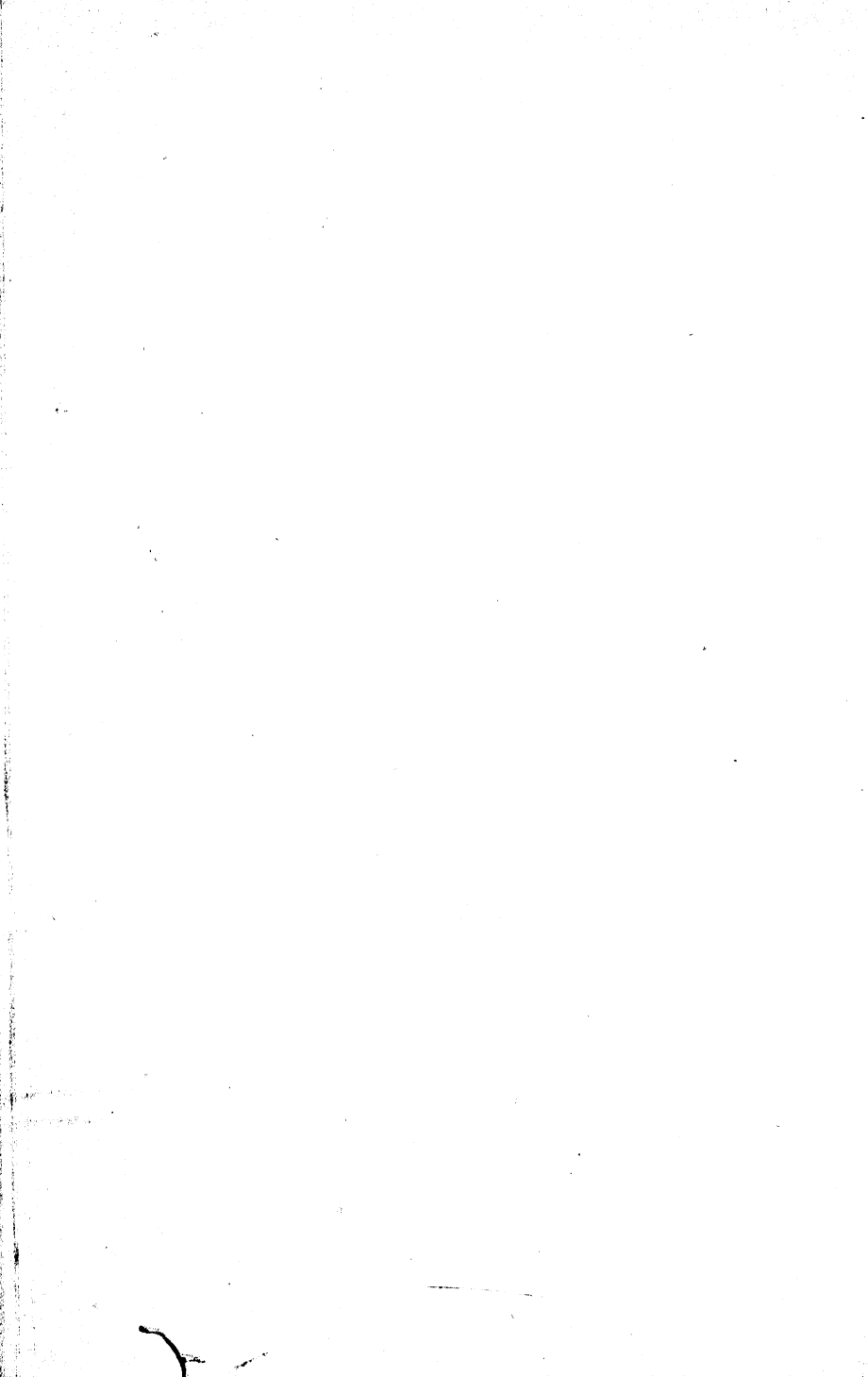
A fixative containing formol and alcohol, and coating of the slides

²In our technic previously reported (1947) we used alcoholic silver solutions and complexes to avoid the use of collodion. Although both types of the technic here recommended can be worked using alcoholic complexes, aqueous ones are more satisfactory and smaller concentrations are required.

with collodion are essential in "direct" reactions, although much less important in "developed" ones.

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CONCENTRATED FORMALIN VERSUS A 10% SOLUTION AS A FIXATIVE PRECEDING SILVER STAINING

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ABSTRACT.—The effect of concentrated and 10% formalin as fixatives for the silver staining of axons in the central nervous system in a series of mammals has been compared. It is felt that concentrated formalin constitutes a better fixative for this purpose in several respects: it has a greater speed of penetration without causing additional distortion or artifacts, the staining time can be reduced somewhat and the normal configuration of the axons is better preserved particularly in the largest ones. Satisfactory results were obtained following 5½ hours fixation in concentrated formalin. Further, concentrated formalin is at least equal to a 10% solution in the ease in which paraffin and frozen blocks can be sectioned, in the clearness of the background of stained sections and in the consistency of results.

The purpose of the present investigation is to compare the possible value of concentrated formalin (40% commercial formalin, or formaldehyde) with that of a 10% solution as a fixative for the silver staining of axons in the central nervous system.

Since 1893, formalin has largely superseded Müller's fluid as a fixing reagent for the tissues of the brain and spinal cord. It is almost universally accepted that it is best employed as a 10% solution of concentrated commercial formalin. In this percentage, it is widely used singly as a fixative in various axonal staining procedures which employ either silver nitrate or protargol.

Some studies have been made on the histological effects of formalin on the tissue of the central nervous system, notably by King (1910, 1913). She tested only solutions of 10% formalin on the brain of the albino rat and found that it caused considerable general swelling. She concluded that it was not a satisfactory fixative to show the cell structure of the brain, as it causes a pronounced distention of the nuclei with poor preservation of the nuclear contents. On the other hand, she stated it does not adversely affect the nerve tracts as determined by the Weigert stain. She did not employ higher concen-

trations of formalin in her experiments nor did she investigate the effect on the axis cylinders by means of silver staining.

Concentrated formalin solution is a fluid which has a high specific gravity, 1.124; its pH is in the acid range, and it is known to be excellent in respect to tissue penetration.

Some of the factors that are of importance in judging the value of any fixing reagent may be the following:

1. Speed and thoroughness of penetration.
2. Degree and consistency of hardening of fixed tissues.
3. The manner in which the tissues section.
4. The subsequent selectivity and degree of sharp staining it permits of specific as well as background elements.

It is not the intent of this investigation to determine the merit of concentrated formalin as a general fixative but merely to compare it with the commonly used 10% formalin in respect to the factors listed in the preceding paragraph.

MATERIAL AND METHODS

About 1,500 sections from 635 paraffin-embedded blocks of nervous tissue have been made from the spinal cords and brain-stems of 18 animals: 2 rabbits, 2 rats, 11 cats, 2 monkeys and 1 human. The blocks varied in length from about 3 to 5 mm.

The times allotted for fixation in non-neutralized concentrated formalin at room temperatures (average, 24°C., 75°F.) were the following: 15, 30, 45 and 60 minutes; 2, 3, 5½, 7, 10½, 12 and 24 hours; 3, 4, 5, 6, and 7 days, and then removal at weekly intervals up to 2½ months. In all the experiments, immersion of blocks for the same durations in 10% formalin were used as controls.

In the majority of instances, fixed tissues were washed overnight in running water, then run up through the alcohols and xylene and eventually embedded in 60–62°C. rubber paraffin. In a limited number of blocks from each animal, dioxane (for 24 hours) was tried as a dehydrating agent.

For the staining procedures, Bodian's protargol method (1937), Davenport's silver nitrate method including gold toning (1930) and the 2-hour staining method with protargol of Davenport *et al* (1939) were used. Except for the minor variations listed below, the above technics as originally presented have been followed.

Davenport's silver nitrate method (1930) for paraffin sections consists basically of impregnating with an alcoholic silver nitrate solution and then reducing in a mixture of pyrogallol, formalin and ethyl alcohol. After reduction by the above method, we placed

slides in a 5% solution of sodium thiosulfate for 5 minutes to eliminate any possible residual salts.

In the technic for the staining of axons with protargol developed by Davenport et al (1939), we obtained better results by cutting down the time from 1 hour to 20 minutes while the sections were being impregnated in both 10% aqueous silver nitrate and 0.2% protargol solutions.

RESULTS

Grossly, a difference could be detected between the fixing reaction of concentrated and 10% formalin on tissue of the central nervous

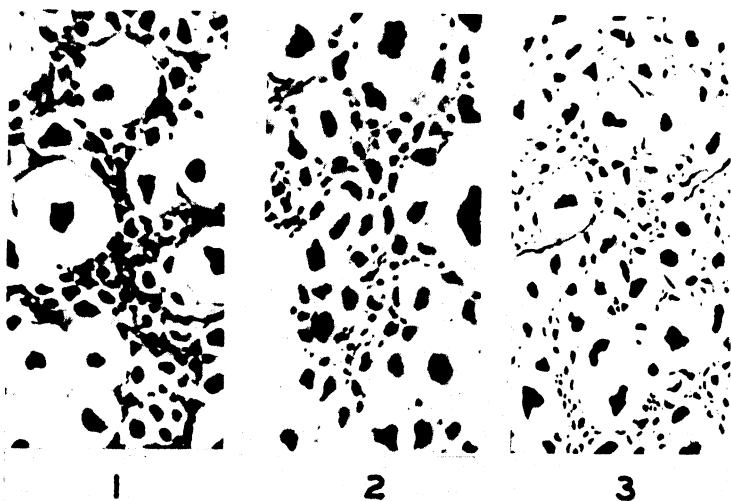


Fig. 1 and 2 show the silver staining response of axons in the anterior funiculus of the spinal cord of the cat following $5\frac{1}{2}$ hours and 5 weeks fixation in non-neutralized formaldehyde (40% commercial formalin) respectively. Staining: Davenport's silver nitrate method followed by gold toning.

Fig. 3 shows the axonal reaction in the anterior funiculus of the spinal cord of the rabbit following $5\frac{1}{2}$ hours fixation in formaldehyde. Staining: Bodian's activated protargol method.

system. Because of the high specific gravity of the former, the blocks float or remain near the top surface of the fluid at first, but within 2 to 3 hours they gradually fall to the bottom. The thoroughness of penetration can, to some extent, be correlated with the sinking of the blocks. The same is not true for 10% formalin as the specimens sink immediately upon immersion. The 10% solution

causes the cut ends to become very fuzzy for a week or so. The material which accumulates on the cut ends then drops off and forms a sediment in the fluid. In contrast, the specimens fixed in concentrated formalin retain an almost glassy smoothness throughout the duration of submersion. It may be that this is the key to an understanding of the different results we have obtained between the two mediums under consideration.

The penetrating power of concentrated formalin is much more rapid than that of 10% formalin. Small axons will stain throughout the cord after only 15 minutes fixation in concentrated formalin. After 5½ hours fixation in the same fluid, both large and small axons are well differentiated (Fig. 1 and 3), and this time is considered to be near the minimum necessary to harden the specimens adequately although very satisfactory preparations were often obtained after 3 hours fixation. Prolonged submersion, up to 10 weeks in concentrated formalin, does not seem to alter the staining response.

In all of the blocks of cord and brain-stem which were sectioned, it was found that those fixed in concentrated formalin cut just as easily and satisfactorily as those subjected to the action of 10% formalin. No difficulty was encountered at any time in respect to ribboning, flattening of the sections, or in tearing and distortion of the tissues.

The main advantage of concentrated formalin-fixed tissues, as far as staining is concerned, appears to be that it is followed by much less shrinkage of the axis cylinders and particularly of the largest ones. Much better results were obtained with Davenport's silver nitrate method (1930) and with Bodian's technic (1937).


The cells also seem to undergo less shrinkage and distortion following fixation in concentrated formalin. Although no direct effort was made to stain any of the peripheral nerves, it was noticed that the dorsal and ventral roots responded well and perhaps better following fixation in concentrated formalin.

Dioxane, as a dehydrating agent, works satisfactorily following fixation in concentrated formalin but the axons appear to have slightly less affinity for the silver stains.

A number of attempts were made to test the ease of cutting tissues fixed in concentrated formalin with the freezing microtome. Tissues which were fixed from 2 to 24 hours and then frozen, sectioned, as nearly as we could judge, about the same as those subjected to 10% formalin.

COMMENTS

On the basis of the results obtained, it seems that concentrated formalin constitutes a reagent which may serve as a better fixing



medium than the diluted 10% formalin for the staining of axis cylinders in the central nervous system of mammals. We believe that it is superior in several respects. It has a much more rapid penetrating power without causing any additional distortion; it affects the tissues so that the staining time can be reduced somewhat and it allows better preservation of the normal configuration of the axons and particularly of the largest ones. It is equal to, or may excel, 10% formalin in the following: in the ease of sectioning paraffin and frozen blocks, in the clearness of the background picture in the staining procedure, and in the consistency of results.


We are not in a position to say what the phenomenon may be which occurs on the cut surface of cords placed in 10% formalin. The characteristic fuzzy appearance, which is not seen in specimens fixed in concentrated formalin, may be due to swelling of the myelin or to some other factor or factors. Whatever the accumulated material at the cut surfaces may be, it is eventually liberated in a week or two to form a sediment in the fluid medium.

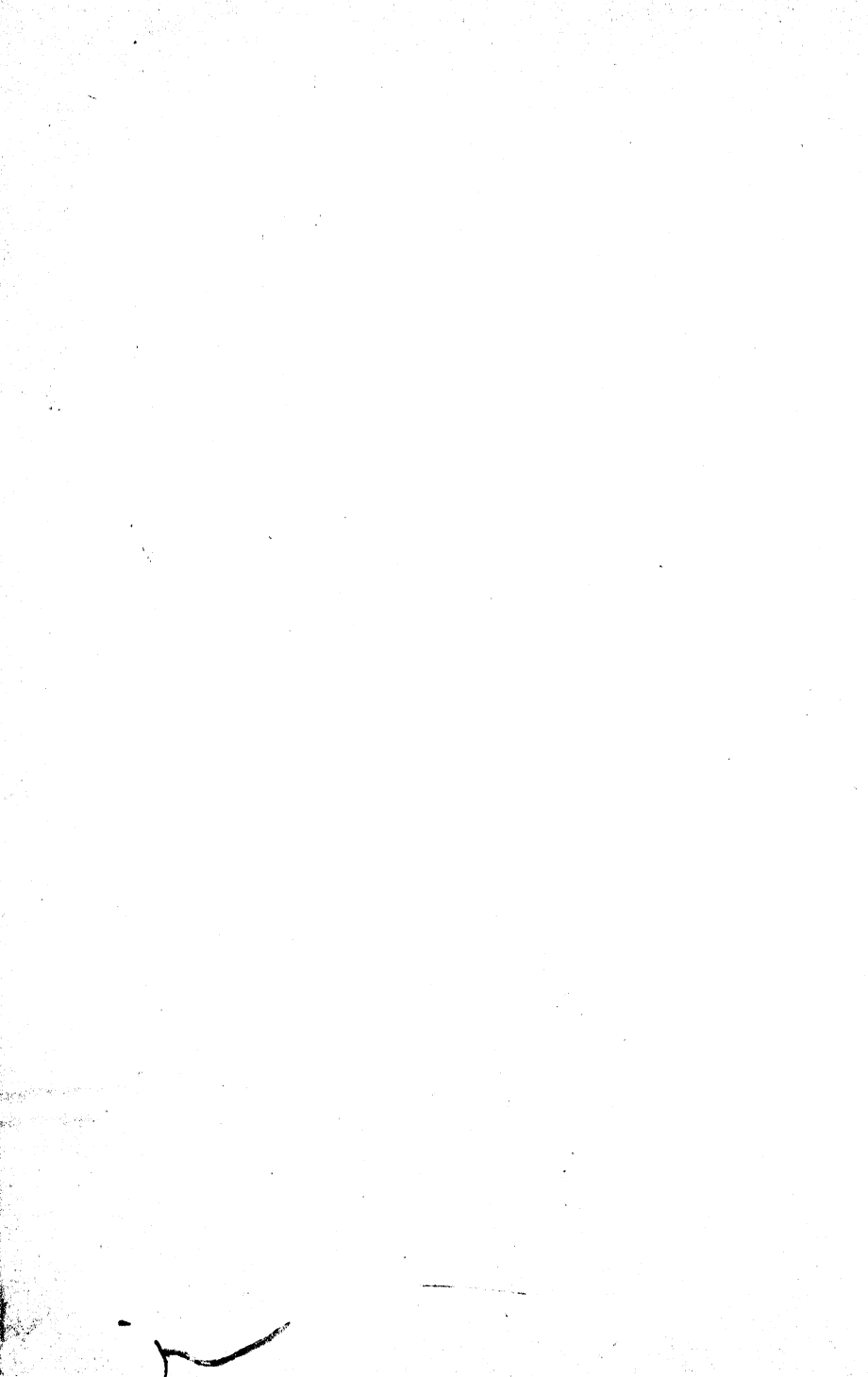
It is not claimed that concentrated formalin constitutes a perfect fixative for the staining of axons with silver compounds. However, in our hands, it has been judged to be consistently superior to 10% formalin and our results have been such that further experiments are in progress.

CONCLUSIONS

In a series of experiments on the brain-stem and spinal cord of the rat, rabbit, cat, monkey and man, concentrated seems to excel 10% formalin as a fixative for the staining of axons with silver nitrate or protargol compounds. It has proved to be better in respect to speed of penetration without causing additional distortion, in allowing a slight reduction in the staining time and in permitting better preservation of the normal configuration of the axons and particularly of the largest ones. Further, it is at least equal to 10% formalin in the ease in which paraffin and frozen blocks can be sectioned, in the clearness of the background of stained sections and in the consistency of results.

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BRILLIANT CRESYL BLUE AS A STAIN FOR PLANT CHROMOSOMES

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ABSTRACT.—Methods are proposed for staining plant chromosomes with the dye brilliant cresyl blue, and for making these stained preparations permanent by using polyvinyl alcohol mounting medium.

The stain, which is composed of 2% brilliant cresyl blue in 45% aqueous acetic or propionic acid, is used with fixed material in making smear preparations. The technics for staining are similar to those employed in the aceto-carmin method.

The mounting medium is made by mixing 56% polyvinyl alcohol, which is diluted in water to the consistency of thick molasses, with 22% lactic acid and 22% phenol by volume. The permanent slides are made by floating off the cover slip of the temporary slide in 70% alcohol, then applying the mounting medium and replacing the cover slip.

The chief advantages of the methods described are:

- 1) The preparation of the stain is rapid and simple. The batch of stain will be good with the first try.
- 2) The staining procedure in some instances is shorter than when using aceto-carmin.
- 3) The stain shows a high degree of specificity for nuclear structures and gives better results than aceto-carmin when used on certain plant tissues.
- 4) A minimum number of cells is lost in making the slides permanent when using polyvinyl alcohol mounting medium as the slide and cover slip are run through only one solution prior to mounting.
- 5) The mounting medium dries rapidly and this shortens the time required before critical examination of the permanent mounts can be made.

INTRODUCTION

In attempting to make a quick determination of the number of divisions occurring at a given time in the root-tip cells of *Phaseolus aureus*,¹ the standard aceto-carmin method was tried with poor

¹*P. aureus* is being used as an experimental plant by Dr. H. Quastler, Mrs. R. Baer, and the authors to test radiation effects.

results. A modification of the brilliant cresyl blue method, used as a stain for the salivary-gland chromosomes of *Drosophila macrospina macrospina* (Mittler and Bartha, 1948) was then applied to *P. aureus*, and subsequently to the root tips of *Allium*, *Zea*, and *P. vulgaris*, and the anthers of *Rhoeo*.

The stain. Brilliant cresyl blue is a basic dye which has served in the past as a vital stain and as a stain to bring out reticulated blood corpuscles (Conn, 1940). The preparation of this stain, as used in the following technics, is a simple procedure. Two per cent brilliant cresyl blue² is dissolved in 50% aqueous propionic acid and filtered. Equally good results are obtained with 45% glacial acetic acid in place of the propionic acid. It is not necessary to boil and decant as in the preparation of aceto-carmine (Smith, 1947), nor is it necessary to add iron. It has been found that this acid solution of the stain brings about distinctly more selective staining of the nuclei than either plain aqueous or plain alcoholic solutions. There is little chance that the stain will not be satisfactory with the first attempt.

The stain, prepared in the manner described above, has an ink-like density. However, this does not prevent microscopic examination of the cells on the slide during the staining process. It is possible to see the cells clearly at the periphery of the drop of stain prior to the application of the cover slip.

The chromatin of the interphase nuclei shows a striking affinity for the stain in contrast to the nucleoli which do not stain at all (Fig. 2, 4). Chromosomes may retain a degree of translucency after staining which reveals their structure at various stages of division. These nuclear structures are stained red-brown to nearly black, while the cytoplasm remains colorless or nearly so, depending on the plant tissues used (Fig. 1-8). In temporary slides the colorless cells with their dark-staining nuclei appear in sharp contrast against the blue background.

With illumination from an ordinary microscope lamp a white diffusing filter is desirable plus a blue filter if the stain is light.

Fixation. All the plant tissues tried stained in 2% brilliant cresyl blue alone, but the resulting fixation was poor. The best results were obtained by staining material which had been fixed in 3 parts of 95% ethyl alcohol and 1 part of glacial acetic acid (Farmer's fluid), or the above modified to contain 2 parts of 95% alcohol and 1 part of glacial acetic acid.

²The lots used are old and bear no certification numbers. They are products of the Coleman and Bell Co., Norwood, Ohio.

ROOT-TIP SMEARS

The technic used in making root-tip smears of *Allium* served as a basis for making similar preparations of *Phaseolus* and *Zea*.

After fixation, the root-tip smears of *Allium* are made as follows:

- 1) Place the root tip in a vial or preparation dish containing 1 part of concentrated hydrochloric acid and 1 part of 95% ethyl alcohol. Maceration should be complete at the end of 5 minutes.
- 2) Transfer the root tip to Farmer's fluid for a period of 2 to 3 minutes.
- 3) Place in 70% ethyl alcohol for 2 to 3 minutes.
- 4) On the surface of a clean slide which has been coated with a thin film of Haupt's adhesive place a small drop of stain. Remove the root tip from the 70% alcohol and with a scalpel or razor blade cut cross-sections from the terminal millimeter of the embryonic region. Push these into the stain.
- 5) Allow the staining process to go on for 3 to 5 minutes, using the scalpel or razor blade during this interval to separate the cells further. At the end of this time examine the cells which accumulate at the margin of the drop of stain to determine the degree of staining.
- 6) Apply a clean cover slip and remove the excess stain from its margins with a paper towel or blotter. Cover the slide with the paper towel and apply direct thumb pressure on the area of the cover slip, being careful not to let the cover slip move.
- 7) Seal the slide with a mixture of equal parts of gum-mastic and hard paraffin.

The staining time for *Zea* and *Phaseolus* must be increased to about 10 minutes. The interphase nuclei of *Phaseolus* (Fig. 6) do not take as deep a stain as those of *Zea* and *Allium* (Fig. 2), but the mitotic figures stain deeply (Fig. 5, 6) and are in sharp contrast to the light background.

SMEAR PREPARATIONS FOR MEIOSIS

The anthers of *Rhoeo*, which had been fixed in Farmer's fluid, were used in the technic which follows:

- 1) Place the flower on a clean slide, the surface of which has been coated with a thin film of Haupt's adhesive. Add a drop of Farmer's fluid to prevent the flower from drying and dissect out one or more of the anthers over a dark background.
- 2) Remove the excess fixative with a blotter or towel and add a small drop of 2% brilliant cresyl blue.

Plate 1

FIG. 1.—Anaphase in a root-tip cell of *Allium*. Note the light staining cytoplasm. $\times 1300$.

FIG. 2.—Root-tip smear of *Allium*. This preparation was made permanent by the polyvinyl alcohol method. $\times 750$.

FIG. 3.—Late prophase during the first division of the pollen-mother-cells of *Rhoeo*. The darker background is due to the presence of the stain in the temporary slide. $\times 1300$.

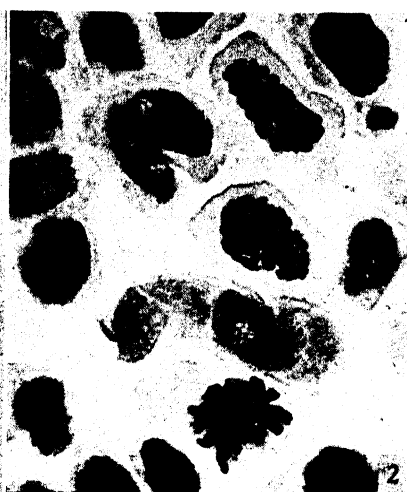
FIG. 4.—Temporary preparation of a root-tip smear of *Allium*. Note the lack of stain in the nucleolus and cytoplasm as contrasted to the dark stain of the chromatin and anaphase chromosomes. $\times 1300$.

FIG. 5.—Metaphase in a root-tip cell of *Phaseolus aureus*. This slide was made permanent by the polyvinyl alcohol method. It was a month old at the time the photograph was taken and there was no sign of fading. $\times 750$.

FIG. 6.—Prophase and metaphase in a root-tip cell of *Phaseolus aureus*. This preparation was made permanent by Bradley's modification of Bridges' alcohol vapor method. Note that the cytoplasm is darker than in those slides made permanent by the polyvinyl alcohol method. $\times 750$.

FIG. 7.—Second division prophase in a pollen-mother-cell of *Rhoeo*. This slide was made permanent by the polyvinyl alcohol method. $\times 750$.

FIG. 8.—First division anaphase in a pollen-mother-cell of *Rhoeo*. The treatment is the same as described above. $\times 750$.



- 3) Break open the anthers in the stain and with a small scapel squeeze the pollen-mother-cells out.
- 4) After 4 minutes of staining, examine the cells which have accumulated at the periphery of the drop of stain. If the stain is not dark enough, allow a longer time.
- 5) If the stain is dark enough, remove the debris and put a clean cover slip in place. Cover with a paper towel and apply direct, gentle thumb pressure.
- 6) Seal with the gum-mastic-hard-paraffin mixture.

The structure of the meiotic chromosomes of *Rhoeo*, stained with brilliant cresyl blue, is well defined (Fig. 3, 7, 8).

MAKING PERMANENT SLIDES

After the first temporary slides made were about a week old, it was noted that the stain had started to precipitate. In addition, the stain faded from the *Phaseolus* and *Zea* preparations. These two difficulties were overcome by making the slides permanent shortly after the cover slip was first applied.

Of the technics tried for making permanent slides, two were found to be more successful than the others. The first of these is the modification of Bridge's alcohol vapor method as reported by Bradley (1948), which was selected as a technic to eliminate loss of cells. The second technic involves the use of a plastic mounting medium according to the formula given by Huber and Caplin (1947). They give the following instructions for its preparation:

- 1) 15 g. of polyvinyl alcohol ("Elvanol" 71-24)³ is added slowly to 100 ml. of cold water.
- 2) Stir and heat in a water bath to 80°C. until it has the consistency of thick molasses.
- 3) Filter if necessary with a cheesecloth.

The complete mounting medium consists of 56% of the stock solution to which is added 22% lactic acid and 22% phenol by volume. This mixture dries rapidly and there is a tough seal at the periphery of the cover slip after 12 to 18 hours. The excess dried mounting medium is removed with water.

After staining with brilliant cresyl blue as previously outlined, the mounting medium is applied as follows:

- 1) The slide is supported in an inverted position by glass rods in a Petri dish (staining dishes, etc., will work as well). The Petri dish is filled with 70% ethyl alcohol so that the slide is

³This grade of polyvinyl alcohol may be obtained from E. I. du Pont de Nemours Co., Electrochemicals Department, 7 S. Dearborn Street, Chicago, 3, Illinois.

immersed and the cover slip is allowed to float off to the bottom of the dish.

- 2) Remove the excess 70% alcohol from the slide and cover slip by touching their edges to a paper towel.
- 3) Apply a very small drop of polyvinyl alcohol mounting medium and replace the cover slip in exactly its former position.

DISCUSSION

The methods described above for staining plant chromosomes with brilliant cresyl blue and for making slides permanent with polyvinyl alcohol mounting medium are of value in that the total time required is shorter than when using other technics.

The preparation of the stain takes only a matter of minutes. The time required for making the temporary smear preparations is about the same as for the aceto-carminic technic. The chief advantage in using brilliant cresyl blue as a stain is that in certain plant tissues it gives better results than aceto-carminic. For this reason it is thought that the brilliant cresyl blue technic may serve as an added tool for cytological use.

After staining with brilliant cresyl blue, the polyvinyl alcohol method for making slides permanent has two advantages. First, the loss of material from the slide and cover slip is minimized. Second, the time required is shortened as the mounting medium dries rapidly and the slides are ready for critical observation after 12 hours.

ACKNOWLEDGMENT

The authors wish to thank Mr. Alexander Bartha for suggesting the use of this dye on plant materials.

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TREATMENT OF *TRILLIUM ERECTUM* PRIOR TO AND DURING MASS PRODUCTION OF PERMANENT SMEAR PREPARATIONS

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ABSTRACT.—Technics used in handling dormant plants of *Trillium erectum* L. prior to and during microsporogenesis are described. Normal meiosis occurs during storage of the plants at 4–6°C. Approximate times in days required for various stages of meiosis are presented. Schedules used for the mass production of aceto- or propiono-carminic smears of microsporocytes, microspores and pollen tubes are also given.

INTRODUCTION

As material for cytological research and for teaching purposes *Trillium* offers a number of attributes not present in certain other plant species now widely used for these purposes. The present report is presented in response to a number of requests for information on the handling of *Trillium* in the hope that greater use may be made of such an excellent subject for cytological investigations.

The most important factors which make *Trillium* so desirable are (1) a low number ($n=5$) of very large chromosomes (up to 20μ in length), (2) the chromosomes can be distinguished morphologically at metaphase and anaphase (Fig. 1, 4), (3) spiral structure can be readily demonstrated without the use of special technics (see Fig. 1, 2), (4) the anthers are large (4–15 mm. in length) and hence yield a large number of microsporocytes (up to 25,000 for each anther), (5) the plants can be obtained readily from commercial sources¹ and (6) a greenhouse is not required.

GENERAL INFORMATION

The methods given below have been found suitable for *Trillium erectum* L. (ill-scented or purple-flowered wake-robin). The cytological methods are generally adaptable to other species² but probably

¹The Gardens of the Blue Ridge, Ashford, N. C. can supply several species in large numbers.

²The taxonomic literature on various *Trillium* species is reviewed by Barksdale (1938). A few references to cytological investigations are also given.

not the handling of the rhizomes since a number of species (e.g. *T. grandiflorum*, *T. luteum* and *T. undulatum*) complete their meiosis in the fall and have a very long postmeiotic resting stage. Microspore mitosis in such species would be expected to occur in the early spring.

For those who wish to collect their own material³, it is suggested that they do some preliminary field work in the spring, when the plants are in bloom, to locate and mark an area which contains a sufficient number of plants. This is advisable because the plants are much more difficult to locate during the fall season. Colored stakes or tags can be used to mark different species where more than one species is present. In the northeastern United States and adjacent provinces in Canada, the rhizomes should be dug in late September or October. This date is also a suitable time to order from dealers if material is not available locally. The delivery date can be specified to insure material at the desired time. If plants are purchased, it is advisable to specify large size rhizomes or at least to specify plants containing undamaged flower buds (small rhizomes usually do not contain flower buds).

Mature plants of *Trillium erectum* L. removed from the ground from late September to November usually contain well developed flower buds. The anthers contain microsporocytes in the premeiotic resting or early meiotic prophase stages. When the plants are kept at a temperature slightly above freezing, meiosis will usually take place in two to four months. Normal meiosis apparently will not occur without exposure of the plant to low temperature. In practice, 4-6° C. has been found to be the most satisfactory for storage of the dormant plants. The onset of meiosis can be controlled to some extent by altering the time at which plants are initially placed in cold storage. Plants placed in refrigeration in October will reach meiosis earlier than those put in during November. Where a large number of plants are needed, this is a useful method of prolonging the season when meiotic stages are available.

For cold storage purposes, it is not necessary to plant the *Trilliums* in soil. They will do much better and be more readily accessible if they are simply placed in a glass or other covered container with some moist sawdust, peat moss, vermiculite, etc. to reduce loss of moisture.

If root tips are desired, the rhizomes may be planted in flats in the greenhouse and dug up when tips are needed, or they may be placed in a moist chamber at room temperature. *Trillium erectum* has been

³It may be advisable in some cases to obtain a permit from the appropriate authorities before collecting begins.

found to produce fewer new roots under cultivation than certain other species. *Trillium grandiflorum* (Michx.) Salisb. for instance, will usually produce a fair supply of root tips in four to eight weeks. Growth of root tips from seeds is a slow process owing to the peculiarly slow and uncertain manner in which *Trillium* seeds germinate (Barton 1944). For best results it is advisable to plant the seeds immediately after removal from the plant so that they have no opportunity to become thoroughly dried.

MATERIAL FOR BIOCHEMICAL ANALYSIS

If desired, anther contents may be squeezed out and used for biochemical analysis. Two to ten milligrams of material can be obtained from a single set of anthers. The amount obtained varies from plant to plant and increases considerably in older anthers. A 2-mg. sample, for example, is sufficient for quantitative determination of nucleic acids. (Steele, Sparrow and Ottolenghi, unpublished).

CYTOLOGICAL METHODS

The stages of meiosis are generally well synchronized in the anthers from a single flower bud. There is, however, some variability, especially where stages of short duration are present and there is frequently a detectable gradient from end to end in an anther. These differences are usually small and often barely noticeable but occasionally there is a poor synchronization of stages, both within and between anthers. When more than one bud is present there is usually, but not always, a difference in stage of development. This frequently proves very useful in obtaining a variety of stages.

As a preliminary to smearing, it is often desirable to make a test slide to determine the stage of meiosis. To do this, a longitudinal slit can be made in the side of the bud (Text Fig. 1) and a part of an anther removed and smeared. If the stage is too early, the plant can be replaced in the cold room and checked periodically until the desired stage is reached. At low temperatures, such plants will usually survive for weeks or months even though the incision has been made deep enough to expose the anthers. However, the open ends of cut anthers act as point of entry for bacterial infection or fungal attack and a small proportion of plants is lost.

A convenient method of labelling the rhizomes is to use small plastic plant labels attached with a fine copper wire. String and paper labels are not recommended as they are frequently destroyed by fungi in the moist containers.

The approximate time required for *Trillium erectum* to pass through various stages of microsporogenesis at 4-6° C. is given in Table 1. The extended duration of certain stages is a great advantage where it is desirable to have material available at a particular stage of division, for experimental purposes. In plants which undergo mei-

osis at normal summer (or greenhouse) temperatures, the much shorter duration of most stages makes them more difficult or impossible to use for certain experimental purposes for which *T. erectum* is well suited.

The times given in Table 1 are only approximate times. It should be clearly understood that the deviation from average is high in many cases. This is particularly true if plants have been given an experimental treatment such as exposure to x-rays which tend to inhibit division for a time. It should also be noted the times given are only for low temperatures (around 4–6°C.). Once late meiotic prophase has been reached, later stages can be speeded up considerably by placing the plants at higher temperatures. This holds also for stages of microspore mitosis, but it tends to make the cell walls thicker and more opaque and the chromosomes less condensed at metaphase.

TABLE 1.—APPROXIMATE TIME IN DAYS REQUIRED FOR VARIOUS STAGES OF MICROSPOROGENESIS IN *Trillium erectum* AT 4–6° C.

Stages	Duration in days
Leptotene-zygotene.....	>80
Pachytene.....	18–24
Diplotene-diakinesis.....	12–16
First division.....	5–8
Second division.....	5–10
Post-meiotic interphase.....	28–40
Microspore first division.....	8–14

For certain experimental purposes, *T. erectum* has the advantage that different anthers from the same bud may be removed days or weeks apart and thus cells may be obtained at a number of stages, e.g., at both meiotic and microspore divisions, or at both first and second meiotic division. Control material can likewise be provided by removing a single anther or by removing a whole bud where two or more are present. In the latter case, it is recommended that a portion of the rhizome be left attached to each bud to provide food reserves. Single anthers removed whole at late meiotic prophase will undergo first and second meiotic divisions if handled carefully to avoid crushing and are placed in a moist atmosphere or vial. Whole anthers or microsporocytes suspended in White's nutrient have been successfully cultured by Stern (unpublished). Christensen and Ottolenghi (unpublished) have also grown excised anthers in White's nutrient and found that meiosis would proceed from diplotene to the microspore resting stage.

FIXATION

For ordinary aceto- or propiono-carminc smears, no prefixation is necessary but 3:1 or 2:1 alcohol-acetic may be used if it is not desired to show internal (spiral) structure. Fixation of smears on slides or cover glasses may vary from less than one minute to many hours or even days. If desired, such material may be stored in alcohol, preferably in a refrigerator for a period of weeks or months. Fixation of whole anthers or large sections of anthers is not recommended.

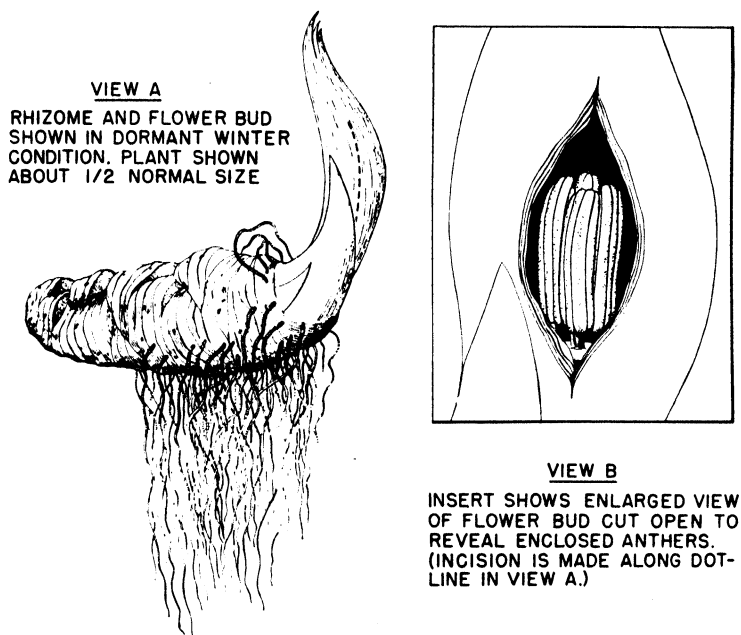


FIG. 1.

SCHEDULE FOR MEIOTIC DIVISIONS

For the rapid production of a large number of slides, the aceto- or propiono-carminc smear technic (see review by Smith) is preferred by the authors. Special procedures for demonstrating spiral structure have been described by Huskins and Smith, Matsuura, Coleman and Hillary and Sparrow (additional references may be obtained from Kaufmann's recent review).

The following schedule has provided satisfactory material for

routine use and has given a fair number of slides showing clear spiral structure (Pl. 1, Fig. 1 and 2):

1. Clean slides are smeared with a small drop of albumin or Haupt's fixative and heated over a low flame. (Many of these can be prepared at one time and stored in staining jars until needed. Cover glasses are also cleaned in advance.)

2. An anther or half anther is removed from the bud and the contents smeared on an albuminized slide. A drop of iron-aceto or propiono-carmin (McCallum's) is added and the cells are gently teased with a fine stainless steel dissecting needle (to prevent over-staining due to added iron). The slide is heated slightly over an alcohol flame, the cover glass added and the slide gently pressed under bibulous paper to remove excess stain. Additional heat and pressing may be applied if a quick examination indicates understaining or insufficient flattening.

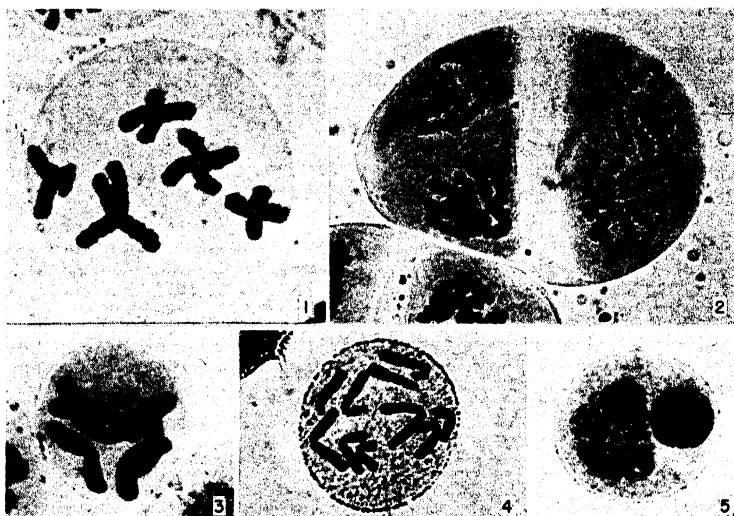


PLATE I

Various stages of microsporogenesis of *Trillium erectum*. Magnification approximately 650 \times . Stained with propiono-carmin. Fig. 1. First meiotic metaphase (with unusually low number of chiasmata). Fig. 2. Second anaphase showing spiralled chromonemata. Fig. 3 and 4. Microspore metaphase and anaphase respectively. Fig. 5. Binucleate pollen grain showing differentiated nuclei.

3. The covers are temporarily sealed with a mixture of paraffin and gum mastic (1:1). [Slides may be kept for several hours longer without sealing if a small amount (3-6%) of glycerol is added to the carmine staining solution].

4. If well flattened cells are desired, a small lead weight (50-100 g.) can be placed on the cover glass.

5. The slides are stored in a cold room or refrigerator for two to four days before being made permanent. The lead weights can be left in place during this period if the slides are handled carefully.

6. If there are only a few slides to be made permanent, they are handled in flat staining dishes or square Petri dishes as follows (modified from McClintock 1929):

- (a) The temporary seal is removed from three sides of the cover glass, but left on the fourth to hold the cover glass in place. Two slides at a time may be placed in 3:1 alcohol-acetic, cover glass side up, for 10–15 minutes.
- (b) If necessary, the cover slips are pried loose gently from the free end, but not removed, in order to insure that the alcohol-acetic mixture reaches all the cells.
- (c) The slides are removed, drained quickly and transferred to 9:1 alcohol-acetic. They remain in this solution for not less than 5 minutes, when the cover glass is again loosened gently.
- (d) The slides are then taken through two changes of absolute or 95% alcohol, remaining in each for at least 2 minutes.
- (e) A drop of diaphane or euparal is added and the cover replaced.
- (f) Excess diaphane is blotted off with bibulous paper and a small lead weight (50–100 g.) is placed on the cover glass to squeeze out excess mounting fluid.
- (g) The weights can be removed after about 2 days but the slides are left flat for about two weeks to dry (less time will be required in a drying oven).

7. When a large number of slides are to be handled, two or three people can work effectively as follows: One person (A) albuminizes slides, cleans cover glasses and runs the previously made slides through the series to make them permanent. A second person (B) smears the anthers on the slides and puts them in 3:1. A third person (C), or B when not otherwise occupied, does the staining, pressing and sealing. If there are a large number of slides to be made permanent, they may be run through the above series in lots of ten or more by using a larger staining dish with removable slide rack. (See also p. 54). In this case, the seal is left along one edge of the cover glass (the edge that will be lowermost after the slide has been placed in the rack). When the first rack of slides is placed in the 9:1, a second set in another rack can be put in the 3:1, and when the second set of slides go into 9:1, a third set can be started in 3:1, etc. This procedure saves a considerable amount of time where many slides are to be processed. An experienced technician should handle 100 slides or more per day.

SCHEDULE FOR MICROSPORE DIVISIONS

Cells in microspore divisions are handled, in general, in the same manner as meiotic cells. The anthers are smeared on previously albuminized slides and may either be stained immediately, or after prefixation in 3:1 alcohol-acetic. Prefixation of 5–10 minutes is sufficient but slides may be left in the fixative as long as 48 hours. Since the microspores tend to stick together in clumps, especially after prefixation, considerable teasing may be necessary after addition of the stain. Microspores are not as easily damaged as meiotic cells and, therefore, may be handled more roughly. After the clumps of cells are broken up as much as possible with a dissecting needle, the cells may be distributed even more by gently sliding the cover glass back and forth (providing there is a slight excess of stain so that the cover glass is floating).

The slide is then heated and pressed, while still hot, under bibulous paper. A small piece of pressed sheet cork, cut the same shape but smaller than the cover glass, is placed on the cover glass and a heavy lead weight (200–300 g.) is placed on the cork. If convenient, the slides may be left under this weight for as much as 30 minutes before sealing or longer if the stain contains glycerol. This aids in flattening the cells. However, care must be taken that too much of the stain does not evaporate.

If more flattening of the cells is desired, this may be achieved by the following method: Heat the slide over a low burner until quite hot (but not hot enough to boil the stain), quickly replace the cork and lead weight and apply firm pressure with thumb on top of the lead weight. Any excess stain should be wiped off. Then seal the cover to the slide with the paraffin-gum mastic mixture and maintain the pressure until the sealing compound has hardened. The slides are stored in the cold room under the lead weights for 4 days or more before being made permanent (a small amount of glycerol in the stain will reduce evaporation and consequent trouble with air bubbles under the cover glass). Fewer cells will be lost than if the slides are made permanent immediately and the intensity of the stain will usually increase during this time.

A method for the rapid production of a large number of similar slides for class use has been developed:

- (1) Lay out 10–20 clean albuminized slides on the table.
- (2) Press out the contents of one or more anthers and stain in a very large drop of carmine.
- (3) Draw up the suspension of stained cells into a pipette with a capillary tip.
- (4) Distribute a small amount of stain and cells to each slide and quickly add a cover glass.
- (5) Add more stain at the edges of the cover glass, if necessary.

The slides may be sealed for temporary use or made permanent in the usual manner. Using this technic, as many as 20 slides can be prepared in less than ten minutes.

The method is especially useful for preparing slides for a large class where it is desirable to obtain many slides as similar as possible with the least amount of effort. It is also useful for getting the largest number of slides from a limited amount of material. When a mixture of stages is desired, the contents of several anthers, each at a different stage, may be smeared together, thoroughly mixed and then transferred by pipette to as many slides as possible.

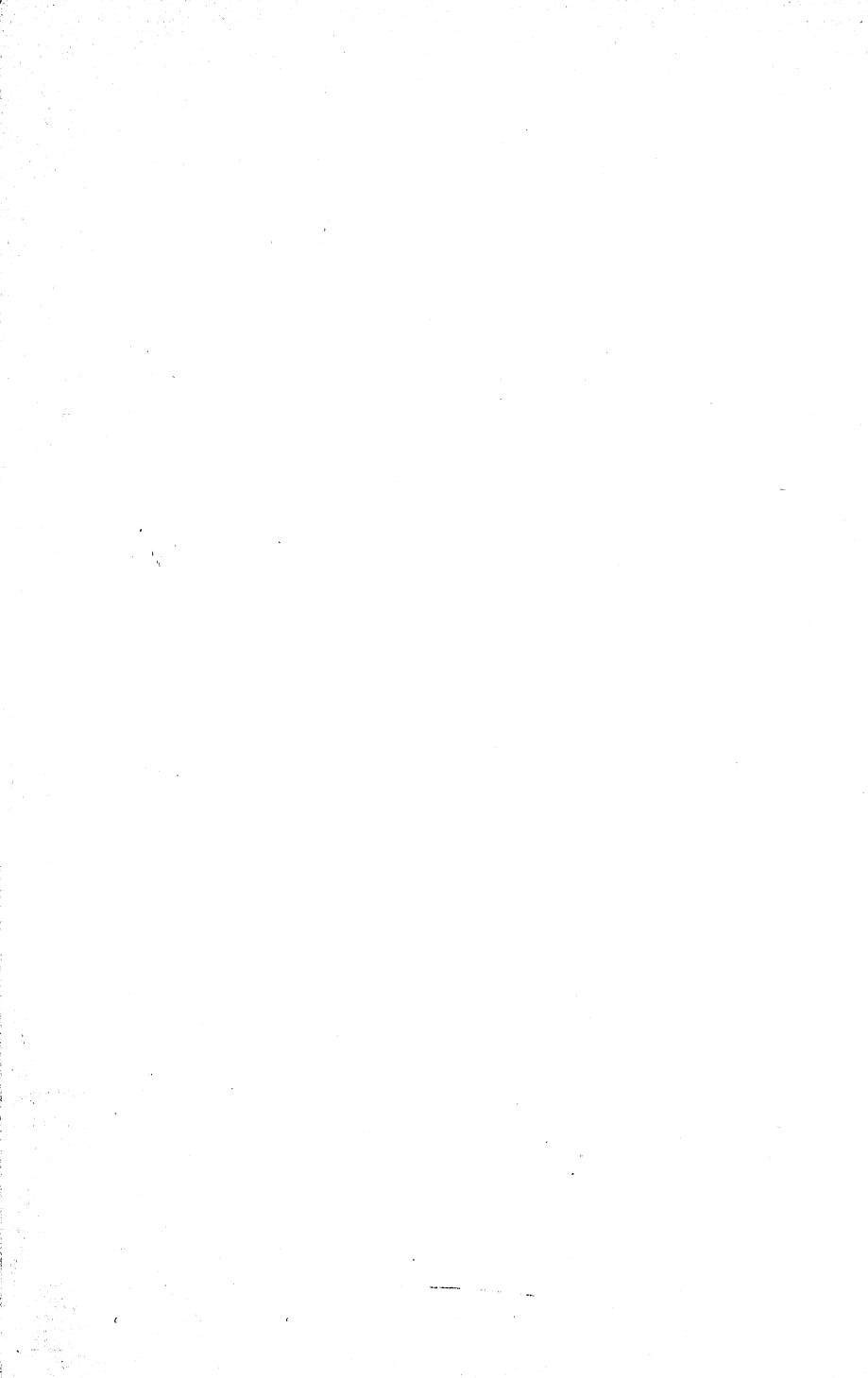
SCHEDULE FOR POLLEN TUBE DIVISIONS

Pollen tubes may readily be obtained by germinating pollen on artificial culture media. Fresh pollen is available for only a brief period under natural conditions but some control of blooming time is possible by varying the length of the time the dormant plants are kept in cold storage. Also, mature pollen may be stored for several weeks under controlled relative humidity (around 50%) and still germinate well enough to give a reasonable proportion of pollen tube division figures.

Many methods of growing pollen tubes have been described, and several of these are satisfactory for growing *Trillium* pollen tubes for cytological study. Bishop's (1948) lactose-agar with or without added colchicine has given excellent results. A warm 1 to 3% agar plus 12% (or less) lactose solution containing 0.01% colchicine is spread in a thin layer on a cover glass and allowed to cool. Pollen is dusted onto the agar medium, and the cover glass is then inverted over a depression slide or ring. Humidity can be controlled by a suitable concentration of H_2SO_4 or saturated by a few drops of water placed in the bottom of the chamber. Metaphase figures should be present after 25-50 hours at normal room temperatures. Later stages will also be present if colchicine is not included in the culture medium.

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A SINGLE SOLUTION IRON-HEMATOXYLIN STAIN FOR INTESTINAL PROTOZOA

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ABSTRACT.—A single solution iron-hematoxylin stain is described for staining fecal smears rapidly and simply. The stain is prepared from the following solutions: Solution A: 1% hematoxylin in 95% alcohol, prepared by diluting a stock solution of 10% hematoxylin in 95% alcohol. Solution B: Ferric ammonium sulfate (violet crystals), 4.0 g.; glacial acetic acid, 1.0 ml.; concentrated sulfuric acid (sp. gr. 1.8), 0.12 ml.; distilled water, 100 ml. Mix equal parts of Solution A and Solution B; allow to stand overnight, filter and use. For maximum length of staining life, store in full, air-tight bottles. To stain fecal smears, fix in Schaudinn's, pass through iodine alcohol to 50% alcohol, stain for three minutes, wash in running tap water 5 to 15 minutes, dehydrate and mount.

INTRODUCTION

In attempting to diagnose intestinal protozoa occurring in feces, it is occasionally found that the direct saline preparation and other temporary mounts are inadequate for the identification of some of the observed organisms. In such cases it may be desirable to fix and stain fecal smears in order to render more visible the morphological features of the various intestinal protozoa. Heidenhain's iron-alum hematoxylin technic is generally recognized as the best stain for this purpose. However, many laboratories working under pressure are prevented from using stained smears for diagnosis because the Heidenhain method is lengthy, and because, in order to obtain proper differentiation, each slide must be handled separately during the destaining process.

To overcome these difficulties, various staining technics have been recommended that do not involve controlled destaining. These have usually employed an alum-hematoxylin like Mayer's solution (Craig, 1942, p. 52) or some hematoxylin-phosphotungstic-acid method as described by Dobell (1942), Ratcliffe and Parkins (1944) and Tompkins and Miller (1947). Iron-hematoxylin stains in the

¹With the technical assistance of Sadie A. Johnson

form of combined mordant and stain mixtures do not seem to have had wide use in the staining of intestinal protozoa, although Weigert's ferric chloride-hematoxylin has been recommended for this purpose² (Thomson and Robertson, 1929, p. 349). Such mixtures are usually less stable than the alum hematoxylin, but the staining effects are often more precise and more nearly permanent.

Other iron-hematoxylin mixtures have been described for the staining of tissue sections in Lee's handbook (1937, p. 155) and by Lillie and Earle (1939), and these may be applicable to fecal smears as well. However, these solutions are not generally stocked by most public health diagnostic laboratories, and their preparation for routine use involves more time than such laboratories can usually spare for the purpose.

The formula to be described here includes, as one component, an acidified ferric alum mordant originally recommended by Lang (1936) for use in the usual, Heidenhain iron-hematoxylin technic. We have found that it may also be used to prepare a single-solution stain that does not require destaining. This fact makes it a convenient reagent for the laboratory that may wish to stain smears either for simple, diagnostic purposes, using the single-solution stain, or for careful, detailed study, using the Heidenhain procedure. The acidification of the mordant with sulfuric and acetic acids improves the precision and clarity with which the single-solution mixture stains intestinal protozoa. The use of the ferric salt alone or in combination with either one of the acids alone does not give as good results as when the solution is prepared as described.

METHODS

Two solutions are used in preparing this stain:

Solution A:

A 1% solution of hematoxylin in 95% alcohol. This is prepared by diluting a stock solution of 10% hematoxylin in 95% alcohol. This solution need not be ripened.

Solution B:

Ferric ammonium sulfate-violet crystals (iron alum)	4.0 g.
Glacial acetic acid	1.0 ml.
Conc. sulfuric acid (sp. gr. 1.8)	0.12 ml.
Distilled water	100.00 ml.

²H. G. Johnstone (University of California, San Francisco) has proposed an iron-hematoxylin mixture for intestinal protozoa that has been adopted in some diagnostic laboratories (unpublished, personal communication).

For use mix equal parts of Solution A and Solution B. The mixture turns a rich purple and overstains when first prepared. Within a few hours the color changes to dark brown with a purplish cast. The solution should then be filtered and may then be used. If the color continues to change until it is greenish-black, the solution is unsuitable for staining. This is often caused by the stock hematoxylin solution's being too ripe and becoming over-oxidized when mixed with the mordant. A freshly prepared hematoxylin solution should then be used instead.

The following staining schedule is recommended:

- (1) Fix smears in Schaudinn's plus 5% acetic acid for 5 minutes at 50°-60° C., or for 1 hour or more at room temperature.
- (2) Wash in iodized 70% alcohol for 5 minutes.
- (3) Transfer through two changes of 50% alcohol for 3 minutes each.
- (4) Stain in above solution for three minutes. This is average time for most smears and may be varied slightly to suit different specimens.
- (5) Wash in running tap water for 5 minutes. If permanence is important, 15 to 30 minutes is better.
- (6) Dehydrate and clear through alcohols, carbol-xylene, and xylene, about 5 minutes each.
- (7) Mount in Clarite or other neutral mounting medium.

Due to the oxidizing influence of the ferric salt used in its preparation the stain lasts about a week when kept in open Coplin jars. When stored in completely filled, glass-stoppered bottles the effective life of the stain is extended to several months. If only a few smears are to be stained at irregular intervals, it is practical to stain them individually on a staining rack. Each slide may then be flooded with only a small amount of solution leaving the rest of the stain in the bottle.

We have found that by replenishing the bottled stain after it has been used a few times, with a small amount of freshly prepared stain, that the entire solution will keep longer and stain more vigorously.

RESULTS

The morphology of the intestinal protozoa stained by this method is the same as that shown by the Heidenhain technic. Ordinarily, the cytoplasm of the organisms will stain more darkly and not as transparently as with the best regressive procedures; in addition the fecal matrix of bacteria and debris is stained less precisely. In the case of *Endamoeba coli* cysts it will occasionally be found that some of

the cysts are refractory and do not take the stain. In those instances one of the regressive technics is usually indicated. In the great majority of specimens, however, nuclei, chromatoid bars, fibrils and other diagnostic features of the various intestinal protozoa are well stained and easily differentiated. When first used the solution stains smears a bright blue. As the solution becomes older, the smears stain progressively grayer.

SUMMARY

(1) The preparation and use of a single solution, iron-hematoxylin stain for fecal smears is described.

(2) The stain is conveniently prepared from stock solutions used in a slightly modified Heidenhain technic.

(3) Intestinal protozoa are easily stained with this method and their diagnosis facilitated.

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THE "DISPERSION STAINING" METHOD FOR THE SELECTIVE COLORATION OF TISSUE

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ABSTRACT.—A method has been developed for the selective coloration of fixed tissue without the use of dyes. Microtome sections of formalin-fixed material are mounted under a cover glass in a mixture of two liquids such as diethylene glycol monobutyl ether with cinnamaldehyde and examined with the dark-field microscope. The refractive index of the liquid used for mounting must be of high dispersion and equal or close to the index of the specimen.

Tissue elements, dependent on their refractive index, whether slightly lower, the same as, or slightly above the mounting medium appear colored in shades of blue, red or yellow.

The optical principles involved in this optical dispersion method are similar to those involved in the production of colored light by the Christiansen filter.

Histological preparations previous to microscopic examination are usually stained by one of the following methods: (1) vital staining, (2) supravital staining, (3) staining after fixation. Vital staining applies to the specific coloration of certain constituents of living tissue chiefly of the reticuloendothelial system and is probably a simple process of phagocytosis of small particles including those of colloidal size such as lampblack, carmine and trypan blue or the filling of vacuoles with particles of colored matter. Supravital staining is the coloring of tissue elements with non-toxic dyes immediately after removal from the body, such as the staining of mitochondria with Janus green and the neutrophilic, basophilic and eosinophilic granules of leucocytes with neutral red. Staining after fixation is the coloration with dyes of protoplasm following its coagulation, precipitation or chemical combination with fixatives such as formaldehyde, ethyl alcohol, picric acid etc. or with mixtures of these chemicals in correct proportion to best preserve both nuclear and cytoplasmic elements. The mechanism of supravital staining and staining after fixation is usually explained as a process of absorption, adsorption or chemical combination between certain tissue elements and dyes. Specific examples can be given to illustrate each of these theories but most

staining is probably a combination of all three, accompanied by such forces as capillarity and osmosis.

It is the purpose of this paper to present more detailed information concerning another method (Crossmon, 1948) for the coloration of protoplasm without the use of dyes by making use of the refractive indices of the fixed tissue. The technic described is based on the use of the dark-field microscope for the examination of tissue mounted in a liquid equal to or close to its refractive index for sodium light. As observed with dark-field illumination, tissue elements differing widely in index from the mounting medium appear white due to the refracted and reflected white light entering the microscope objective. If the difference between the element and immersion liquid is small, certain portions of their dispersion curves will approach coincidence and light of the corresponding wave lengths will be transmitted straight through or slightly refracted oblique to the optic axis of the microscope and thus not enter the objective. The remaining light for which the object and immersion liquid differ to a greater extent in index is refracted and reflected into the objective. The eye then sees the tissue element as colored, the color being the result of the subtraction of the wave lengths passing straight through or slightly refracted from white light. The method is as follows:

1. *Fixation of Tissue.* Ten per cent neutral formalin in 0.85% NaCl was used since it is best that the fixative employed adds no color to the tissue elements as would be the case with mixtures containing picric, chromic or osmic acid.
2. *Dehydration, Embedding and Cutting.* The alcohol-anilin-oil method of dehydration and clearing followed by xylene previous to paraffin infiltration was used. Tissue was transferred through three changes of 50–52°C. paraffin over a period of approximately 18 hours and sections cut at 12 μ .
3. *Affixing Sections.* The usual affixing methods with Mayer's albumen, gelatin or celloidin should not be used since these materials microscopically appear white or colored dependent on the refractive index fluid used for mounting. Sections should be made to adhere to perfectly clean slides without the intervention of any cementing substance, being brought into contact with the glass by evaporation of the distilled water on which they are floated.
4. *Mounting Media.* The mounting media were chosen from a series of liquids differing in index by 0.004 that were equal to or close to the index of the material to be examined and had a much greater dispersion. For best results the liquid should

have low vapor pressure, little or no color, and as applied to tissue preparations, miscibility with xylene. Mixtures of diethylene glycol monobutyl ether and α -chloronaphthalene (index range 1.440 to 1.628), α -chloronaphthalene and α -bromonaphthalene (index range 1.632 to 1.656) and α -bromonaphthalene with methylene iodide (index range 1.660 to 1.70) have been used with success for the examination of many mineral and chemical preparations but have not proved to be ideal for most tissue work. Mixtures of diethylene glycol monobutyl ether (Eastman Kodak Co.) with cinnamaldehyde (Eastman Kodak Co.), being of higher dispersion, were found to be much superior for histological preparations especially for the production of strong coloration when using the higher magnifications such as the $43\times$ achromatic objective. The quantity of the lower index liquid to be mixed with the higher index liquid to give the desired intermediate index can be roughly determined by the equation

$$V_1n_1 + V_2n_2 = V_xn_x$$

where V represents volume, n the index and V_xn_x the volume and index desired. For accurate results it is necessary that the index liquids be checked with a refractometer equipped with temperature regulation apparatus at 25°C . and corrections made by adding the necessary quantity of the lower or higher index liquid.

5. *Equipment.* The microscope and accessories are similar to those described in a previous publication (Crossmon and Gallasch, 1947). The table below gives the optical combinations that have proved successful with a biological type microscope for the examination of microtome sections of histological material. Somewhat inferior results were obtained when using the $10\times$ or $43\times$ achromatic objectives with the Abbe condenser.

<i>Objectives</i>	<i>Condenser</i>	<i>Stop Size</i>
$10\times$ apochromatic (16 mm.), 0.30 N.A.	Achromatic, 1.40 N.A. Top element removed to give 0.59 N.A.	13-14 mm.
$20\times$ achromatic (10.25 mm.) 0.40 N.A.	As above.	16-18 mm.
$43\times$ achromatic (4mm.), 0.65 N.A. with funnel stop.	As above.	16-18 mm.
$10\times$ achromatic (16 mm.) 0.25 N.A.	Abb�, 1.25 N.A. Top element removed to give 0.30 N.A.	16-17 mm.
$43\times$ achromatic (4mm.), 0.65 N.A. with funnel stop.	As above.	22 mm.

The light source should be very bright. A research microscope lamp having a 6 volt 108 watt ribbon filament bulb is satisfactory. For best results it is important that a daylight glass, 50 mm. square, polished on both sides for maximum transmission be used as a filter and that the lamp be equipped with an iris diaphragm.

6. *Microscopic Examination.* Previous to examination with the microscope, the microtome section should be placed in two or three changes of dust-free xylene to dissolve the paraffin completely. The xylene surrounding the tissue should be wiped away with a lint-free cloth and just before the section is entirely dry it should be covered with the desired index fluid and a cover glass applied. The preparation is now ready to examine using first a $10\times$ compensating eyepiece in conjunction with the $10\times$ apochromatic objective. The upper element of the achromatic condenser should be removed, thus reducing its numerical aperture to 0.59 and a dark-field stop 13 to 14 mm. should be placed in the stop holder below the iris diaphragm. It is important that the plane microscope mirror be used and that the microscope lamp be adjusted to produce a parallel beam of light. The condenser should now be racked up or down as may be necessary until the preparation appears bright on a dark background. Increased magnification can be obtained by substituting $15\times$ or $25\times$ compensating eyepieces for the $10\times$ or the use of the $20\times$ and $43\times$ objectives. If the $43\times$ is used, its numerical aperture must be reduced by the insertion of a funnel stop screwed into the objective far enough as to just touch the back lens. A funnel stop with overall length of 1.092 inches and aperture of 0.116 inches has proved to be satisfactory for most preparations.

It is important that the lamp iris diaphragm be carefully adjusted especially when using the $43\times$ objective to prevent internal reflections which often cause a haze over the field of view. It should be partially closed and then opened until it just clears the field of the eyepiece.

RESULTS AND DISCUSSION

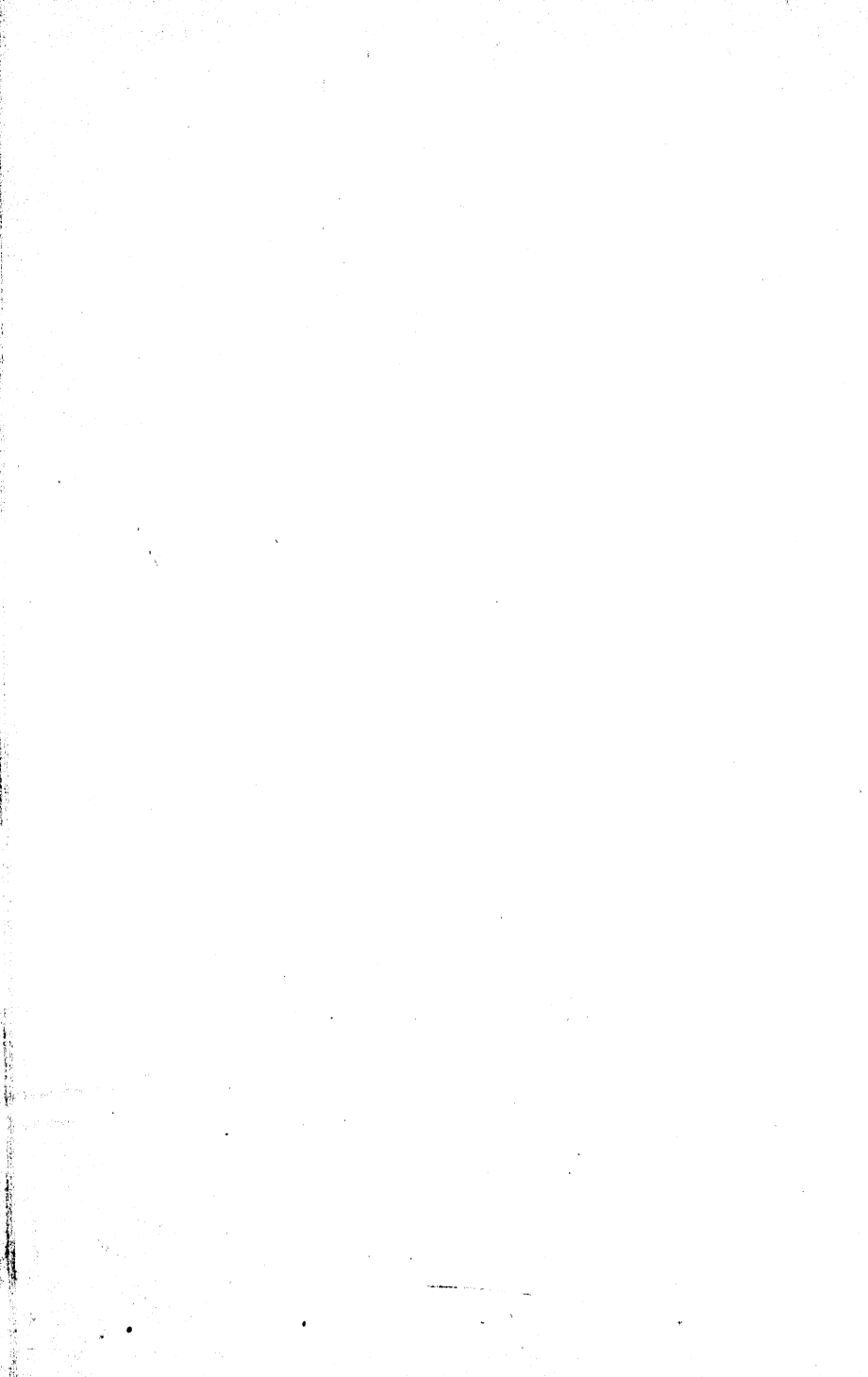
The results obtained are dependent on the refractive index of the tissue elements examined and the liquid used as the mounting medium. Provided the index of the tissue and fluid differ greatly, the microtome section will appear white on a dark background as is commonly observed with dark-field illumination. However, if

the index fluid has a much higher dispersion than the tissue, as is usually the case, and is close to it in refractive index, those elements formerly appearing white will be colored blue, red or yellow depends on their index, whether slightly lower, the same as or slightly above the mounting medium used.

The range of refractive index liquids suggested in this paper is much lower and higher than will be commonly necessary for histological preparations fixed in 10% neutral formalin. It is probable that index liquids slightly above or below 1.536 prepared from mixtures of diethyl glycol monobutyl ether with cinnamaldehyde will be of chief use. Increased accuracy can be obtained by adjusting the index liquids mainly used to a difference of 0.002 and examining the preparation microscopically in a temperature controlled room at 25°C.

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CROSSMON, G. C., and GALLASCH, B. G. 1947. Counting of blood cells by dark-field illumination. *J. Lab. and Clin. Med.*, **32**, 206-9.



LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

COLE, V. W. Polarized light in photo-microscopy. *J. Biol. Photo. Assoc.*, **16**, 147-50. 1948.

Polarized light has been used for the examination of anisotropic substances such as the cross striations in muscle, cellular crystallin substances and lipid material. Since the photography of anisotropic material by use of Nicol prisms is somewhat complicated and expensive, the author suggests the substitution of polaroid lenses. One lens, the analyzer, is placed over the eyepiece of the microscope and the other, the polarizer, incorporated in the light source. A microscope having an apochromatic objective lens (3 mm., N.A. 0.95) and a 10 \times compensating eyepiece used in conjunction with a carbon arc light proved to be the most efficient combination. Two different cameras are suggested although any camera with a ground glass back is adaptable. Comparison photographs of motor end plates (gold chloride stain) with and without polaroid lens are shown.—G. C. Crossmon.

CROSSMAN, GERMAIN. The counting of red blood cells by dark-field illumination. *J. Lab. & Clin. Med.*, **32**, 206-9. 1947.

An ordinary binocular microscope can be readily fitted for dark-field illumination and used for counting blood cells, cells in spinal fluid, bacteria, yeasts, molds, plankton, and dust. For blood cells the following procedure is recommended: Dilute as usual and place in a hemocytometer. Adjust a 6 volt 108 watt bulb to give a parallel beam by focusing the image of the bulb on the wall at 6 feet. Use a Corning Daylite Filter. Place a 16 mm. dark-field stop below the condenser. Use a 10 \times , 0.25 \times N.A. low power objective and 10 \times or 15 \times oculars. Rack the substage condenser until the ruling and the cells appear white on a dark background. A 43 \times , 0.65 \times N.A. objective may be used but an approximately 20 mm. funnell stop is then required. Rheinberg color discs may be substituted for the opaque stops, but have no real advantage, although the appearance is spectacular.—John T. Myers.

PEASE, D. C., and BAKER, R. F. Sectioning techniques for electron microscopy using a conventional microtome. *Proc. Soc. Exp. Biol. and Med.*, **67**, 470-4. 1948.

A method for cutting 0.2 μ sections was developed for a standard Spencer rotary microtome. Rat liver fixed by perfusion with 2% osmic acid was doubly embedded with Parlodion and paraffin. The face of the block to be cut was pared down to about 1 mm. square. The microtome was modified by adding a low angle incline to the feed mechanism to give an advance of only 1/10 the calibrated value. The technic is illustrated, and the original article should be consulted for many essential details.—H. A. Davenport.

RICHARDS, OSCAR W. Phase photomicrography. *J. Biol. Photo. Assoc.*, **16**, 29-38. 1947.

The phase microscope shows organisms that are transparent or that have low contrast in sharp outline and with internal detail at full resolution. Instructions are given for use of this instrument for photomicrography. Methods of focusing, the use of a fast flash-tube light for stopping motion in the specimen, and a simple visual step photometer for estimating correct exposure are described. The advantages of stereophotomicrographs made from pictures taken with the phase microscope are discussed, and methods of making them are given.—Elbert C. Cole.

TAYLOR, E. WILFRED. The practical application of phase-contrast to the biologist's microscope. *J. Roy. Micr. Soc.*, 66, 1-8. 1946.

This article is an excellent account of the phase-contrast principle in microscopy. The introduction states clearly the use to which the biologist will put this new accessory. The historical account is complete in dealing with the discovery and later modifications. A very accurate description of the method as applied to the microscope is given. An adequate presentation of the theory strengthens the article considerably. The final portion deals with the adjustments necessary in the use of phase-contrast. Five plates and six text figures illustrate the use and results obtained by phase-contrast as used in biological work.—*Paul G. Roofe.*

MICROTECHNIC IN GENERAL

DAVIS, H. L., and RYNKIEWICZ, H. J. A fiber identification stain. *Ind. Eng. Chem., Anal. Ed.*, 14, 472. 1942.

The dye mixture contains: acid fuchsin (Color Index No. 692), 6 g.; picric acid, 10 g.; tannic acid, 10 g.; and National soluble blue 2B extra (Color Index No. 707), 5 g. The dyes may be ground together and dissolved in hot water or dissolved separately in any order and diluted to 1 liter. Momentary immersion of fibers in the hot solution gives adequate staining, but 2 or more minutes are required for cold staining. A thorough rinsing in water completes the test. Some dyed textiles may be identified without previous bleaching. When pressed wet (after rinsing) between white absorbent papers, a dye mixture characteristic of the color which would have been shown by the undyed fibers is transferred to the papers. Colors shown by common fibers are: cotton or linen, light blue; wool, yellow; raw silk, black; degummed silk, brown; acetate or nylon, pale greenish yellow; cuprammonium, dark blue; viscose, lavender; vinyon, very pale blue. A solution of this dye mixture is made available by Eimer and Amend, New York, N. Y.—*R. T. Whittenberger.*

NICHOLS, J. Improved method for handling photolabile liquids. *Arch. Path.*, 44, 646-7. 1947.

Disadvantages of present methods of storing liquids such as silver nitrate and osmic acid are discussed. A new device, consisting in placing a properly prepared bottle in a black cup, is described. The bottle is first painted black except for a portion at the base somewhat less than that which will be covered by the cup. Then when the bottle is lifted from the cup, the state of the liquid can be determined readily. The flat-bottomed cup need not be removed during pouring.—*Warren Andrew.*

ANIMAL MICROTECHNIC

GRIGGS, FREDERICK C. The chromosome smear technique: a critical review and improvement of method. *J. Roy. Micr. Soc.*, 66, 25-33. 1946.

The following four sections are presented in detail: (1) nature of the problem; (2) problems involved in modern methods; (3) requirements of an efficient technic; (4) consideration of results.

Modern cytological problems are presented in the first two areas. The third section is outlined as follows: A. Reasonably rapid procedure is necessary. B. The technic must be of such a nature that no great loss of material occurs. C. Transparency is stressed. D. Internal optical differentiation is emphasized. E. The stain must give sharp details. F. The technic must not allow changes of any consequence in preparation after immersion in fluid balsam.

The fourth portion goes into specific consideration of results by the author's own technic which is given below:

(1) Smear in the usual way with fresh material and allow to desiccate for a few seconds (blowing assists this process).

(2) Invert slide over following pre-fixative: 80% alcohol, 80 vol.; glacial acetic acid, 20 vol.

Allow to act for 20-30 min. according to material.

(3) Drain and flood with fresh stain-fixative which consists of *Solution A*:

0.5% hematoxylin (about 2-3 weeks old) 1 vol.; 4.0% iron alum (fresh) 0.751 to 1 vol. (or less); absolute alcohol 2 vol., glacial acetic acid 0.5 to 0.75 vol.; with an equal quantity of *Solution B*: 12.5-20% aqueous acetic acid plus carmine to saturation, boiled and filtered. (Concentration of acetic should be approximately near to that of solution A.)

(4) Wash in 50%, then 80%, and finally 100% alcohol, taking care to ensure that the acetic acid is completely cleared from the slide.

(5) At least three changes of alcohol/xylene should be used in ascending concentrations of xylene; but it is undesirable to pass into pure xylene so one should mount from about 60% xylene plus 40% alcohol. *Solution B* can be added in a lower proportion than 50% to the stain-fixative (3), in which case the concentration of alcohol goes up. Stage 2 should be so varied that stages 2 and 3 together are completed in about 25-35 min.; some material appears to require more pre-fixation and less staining than others, but the above schedule has been used to produce the slides from which the photomicrographs were taken, and can profitably be considered a starting point for most work. The stain should be used within 5-6 hours of making up because a precipitate occurs and staining capacity is lost on filtration.

The author's concluding remarks are as follow: "This technic has not proved easy to handle but, when good preparations are obtained, inner detail of chromosomes is revealed in a manner unobtainable by any other method."—*Paul G. Roope*.

HSU, J. H. An improved method for counting blood platelets. *Science and Technology in China*, 1, 54-5. 1948.

It was found that by using Kristenson's method for counting blood platelets, the platelets form clumps, unevenly distributed; or the individuals may enlarge in size and shape so as to become indistinguishable from a clump of aggregated platelets. The author suggests a solution designated as U.C.F. which avoids these defects. The formula is 40% formaldehyde, 0.1 ml.; urea, 10 g.; sodium citrate, 0.5 g.; distilled water, 100 ml. The red cells are completely destroyed, and the platelets are well preserved, no change in shape and size nor in number being detected within one hour after dilution. The platelet count is performed in the usual manner except for the use of a white blood pipette in which blood, obtained from a finger puncture, is diluted 20 times with the fluid. Eighty small squares in the red area are counted and multiplied by 1,000 to give the number of platelets per cubic millimeter.—*M. A. Darrow*.

KRAMER, SOL. A staining procedure for the study of insect musculature. *Science*, 108, 141-2. 1948.

A method for in toto muscle differentiation in housefly larvae and similar forms is described. Procedure is as follows: Bouin's fluid (30°) 8-10 hr.; 50% ethyl alcohol, 10 min.; 70% ethyl alcohol, 1 hr.; 95% ethyl alcohol, 10 min.; 0.5% eosin alcohol, 6-8 hr.; return to 95% alcohol and add oil of wintergreen dropwise to larvae in 95% alcohol at hourly intervals over a period of 4-5 days; transfer to oil of wintergreen. Details of procedure and methods of examination are given in the discussion.—*T. M. McMillan*.

LAECOM, RODNEY C., and CARTER, GEORGE H. Erythrocytes in urinary sediment: identification and normal limits, with a note on the nature of granular casts. *J. Lab. & Clin. Med.*, 33, 875-80. 1948.

The erythrocytes are stained by the following procedure:

Place a drop of sediment on a slide and add a drop of benzidine-nitroprusside solution made by diluting 1.0 ml. of 1.0% aqueous benzidine with 6.0 ml. of water and adding 1.0 ml. of 2% aqueous sodium nitroprusside (stable for about 2 weeks). Mix by tilting. Add 1 drop of 3% H_2O_2 and mix. If the urine is alkaline, add 1 or 2 drops of 10% nitric acid to the sediment in a centrifuge tube, wash 3 times with 0.9% NaCl solution and proceed as above. Erythrocytes are stained a deep blue-purple. If the cell has deteriorated, it will stain light blue. Yeast cells show a blue-green iridescence. Leucocytes have a pale gray cytoplasm and a slightly darker irregularly stained nucleus. The granules of casts have the bluish purple hue of erythrocytes. Fungi and spores, pollen grains, and prostatic bodies

are not stained. The stain is based on the pseudoperoxidase reaction of hemoglobin and benzidine. The nitroprusside reagent is a catalyst. Erythrocytes may occur in normal urine up to 2 per high power field. Formaldehyde is a satisfactory preserving agent.—*John T. Myers.*

STRUGGER, S. Fluorescence microscope examination of trypanosomes in blood. *Canad. J. Res., Sec. E, 26, 229-31. 1948.*

To stain vitally living trypanosomes in blood the following procedure is recommended: Mix a drop of freshly-taken blood with a 1:10,000 solution of acridine orange (3,6-tetramethyldiaminoacridin) made with 0.85% sodium chloride; cover with cover slip; examine with a blue light fluorescence microscope. This microscope may be constructed as follows: for light use a carbon-arc lamp with attached convex lens to produce parallel rays; filter light with a cuvette (2½ cm. thick) filled with a saturated solution of copper oxide ammonia so that only blue light reaches the plane mirror of the microscope; over ocular, insert filter containing an orange glass that absorbs blue light quantitatively but allows green, yellow, and red to pass through unchanged; to focus, use slide on which is placed pulverized anthracene in liquid paraffin. Leucocytes and trypanosomes are visible as motile shining, bright green bodies but the erythrocytes are non-fluorescent and scarcely visible. To stain trypanosomes in dried blood smears: smear blood; dry; fix 2 to 3 min. in methyl alcohol; wash; stain 4 min. in auramine solution, 1 part in 1000 parts distilled water, with 5% liquid phenol added later; wash in distilled water 1 or 2 min.; dry in air; keep slide in darkness. With blue light fluorescence microscope, background is black, erythrocytes shine slightly as dark green circles, and trypanosomes shine with bright golden fluorescence. Contrast is improved if a drop of liquid paraffin and a cover slip are put on stained smear.—*Herbert P. Riley.*

PLANT MICROTECHNIC

FLINT, THOMAS J., and MATZKE, EDWIN B. Recent procedures for the study of three-dimensional shapes of resting cells, and a new method for the shape study of cells in division. *Science, 108, 191-2. 1948.*

Stem tips of *Anacharis densa* (Elodea) were dissected out, killed in CRAF fixative, embedded, and sectioned at 25μ or thicker. They were then stained with the Feulgen technic as outlined by de Tomasi, except that they were hydrolyzed longer (30 min.) in N HCl and stained in decolorized fuchsin for 24 hr. After being rinsed in distilled water the slides were put in a strong aqueous ruthenium red solution for 30 min., quickly rinsed again in distilled water, and run as rapidly as possible into absolute alcohol. They were then stained 1½ min. in orange G in absolute alcohol and clove oil, rinsed in clove oil, put in xylene, and mounted in neutral balsam.

The results were as follows: chromatic figures of dividing cells, deep purple; resting nuclei, less intense; middle lamellae darkened by ruthenium red and spindle fibers and cell plate stained by orange G; cellulose walls, deeply colored.—*T. M. McMillan.*

HITIER, H., and QUIDET, P. Obtention de plantes polyploides par traitement a l'hexachlorocyclohexane et au sulfure de polychlorocyclane. *Compt. Rend. Acad. Sci. Paris, 226, 833. 1948.*

Changes in chromosome number similar to those induced by colchicine were observed in tobacco and onion after treatment with hexachlorocyclohexane and polychlorocyclane sulfate under the following conditions: 8% powdered hexachlorocyclohexane, tobacco, 3 to 4 leaves; 2% powdered hexachlorocyclohexane, tobacco, 8 to 10 leaves; 0.01% powdered hexachlorocyclohexane, roots, onion; 8% powdered hexachlorocyclohexane, base of onion bulb; 0.045% sulfur of polychlorocyclane, tobacco, 8 to 10 leaves; 0.05% sulfur of polychlorocyclane, roots, onion; 14% powdered sulfur of polychlorocyclane, base of onion bulb.—*T. E. Weier.*

MICROORGANISMS

HUNTER, LILLIAN M. A study of the mycelium and haustoria of the rusts of *Abies*. *Canad. J. Res.*, Sec. C, 26, 219-38. 1948.

Infected leaves of *Abies* were examined for mycelia and haustoria of certain rusts using fixatives previously used in a study of spermatogonia. Because of the difficulty of showing clearly the entrance of the haustorium of *Milesia polypodophila* into the cells of the cortex of the stem, the following procedure was substituted: fix infected stems with leaves attached in medium chrom-acetic solution; treat in usual manner and embed in celloidin; section at $7\ \mu$ to $15\ \mu$ on a sliding microtome; take sections through usual grades of alcohols, dissolve in celloidin, and lead to a lactophenol solution in graded solutions from 95% or 70% alcohol; prepare solutions of acid fuchsin, cotton blue, and light green, dissolved separately in lactophenol with just enough stain to give a deep color; drain lactophenol from sections and apply each stain separately by a pipette; cover sections with bell jar and leave overnight; the next morning, wash in lactophenol solution to remove stain; mount with cover glass and a few days later ring with amber cement. The haustoria were clearer than with any other method of staining used. The value of the method was in demonstrating the penetration by the mycelium of the wall of the host cell.—*Herbert P. Riley*.

LEVINE, B. S., and BLACK, L. A. Newly proposed staining formulas for the direct microscopic examination of milk. *Amer. J. Pub. Health*, 38, 1210-18. 1948.

A theoretical discussion is first presented of certain principles of adsorption as they apply to the study of staining procedures for the direct microscopic examination of milk.

Based on the above principles, the authors have developed a two-dip and a one-dip procedure for defatting and staining milk films. The two-dip method consists in defatting films as usual (Standard Methods for the Examination of Dairy Products, 8th Ed.), followed by a one-minute submersion of the slide in a 0.6% solution of methylene blue in 95% alcohol. Their one-dip, water- and acid-free solution consists of certified methylene blue powder, 0.6 g.; ethyl alcohol, 95%, 50.0 ml.; tetrachlorethane, technical, 50.0 ml.; glycerol, C.P., 2.5 ml. Slides are immersed in the solution for one minute.

The above staining solutions yielded maximal bacterial counts of milk, as compared with carbolated methylene blue or the Newman-Tampert stain (Standard Methods, 8th Ed.). A polychrome methylene blue solution yielded materially higher counts than carbolated methylene blue.—*M. W. Jennison*.

STRUGGER, S. Fluorescence microscope examination of bacteria in soil, *Canad. J. Res.*, Sec. C, 26, 188-93. 1948.

To observe living soil bacteria in their native condition, soil was stained with the fluorescent dye, acridine orange (3,6-tetramethyldiaminoacridin). If living cells are stained with this dye (solution 1:10,000, pH 7.5) the living cytoplasm and the nucleus show green fluorescence, but if dead cells are stained the dead protoplasm develops a bright copper-colored fluorescence. The staining of soil is carried out as follows: place 1 g. of sifted soil in each of five test tubes; to each tube add 10 ml. acridine orange solution made with tap or spring water; shake. As soils differ in ability to absorb stain, the same soil must be stained with five different concentrations of acridine orange (1:1000, 1:2000, 1:3000, 1:4000, 1:5000). The most suitable concentration can be determined: it is satisfactory when only a little excess stain remains in the solution after heavy shaking. Generally the soil is stained completely in a few minutes, but it is advisable to wait 5 or 10 min. before observing with the fluorescent microscope. Two methods of preparing slides are used: (1) for qualitative analysis of autochthonic bacteria, a stained soil suspension is strongly centrifuged, the solution on top is decanted, and a small quantity of sediment is well mixed in drop of paraffin oil on a slide and the drop is covered with a cover slip; (2) for counts, a small drop is taken with a needle from the stained and shaken soil suspension, put on a slide, and covered with a cover slip. Examination is by a luminous blue light fluorescence microscope with an oil immersion lens. Paraffin oil is a good immersion fluid.

For counting, a counter of 20μ depth combined with a counting-ruled ocular is used. The particles of soil covered with humus and the particles of humus slime have a dim red fluorescence and the living bacteria are green. The stained bacteria are not killed and can be used for culture experiments.—*H. P. Riley.*

HISTOCHEMISTRY

JAKUES, LOUIS B., BRUCE-MITFORD, MARGARET, and RICKER, ANN G. The metachromatic activity of heparin. *Rev. Canad. Biol.*, 6, 740-54. 1947.

When heparin is added to many dyes containing a free amino group, it causes a change in the color of the dye, shifting its absorption spectrum to a shorter wave length. When 2 mg. of heparin are added to 5 ml. of a 5 mg. % solution of bismark brown, the color changes from orange to yellow. With azure A the change is from blue to red, with brilliant cresyl blue or brom cresyl blue from blue to purple, with Nile blue sulfate from greenish blue to reddish blue, and with neutral red at pH 6.7 from red to orange. With basic fuchsin there is a detectable shift in the red color, and with pyronin and acriflavin the solution loses its fluorescence. These color changes are termed metachromatic reactions.

To determine the metachromatic activity of heparin, the following method was used, based on the measurement in the Lovibond tintometer of the red color developed with azure A (National Aniline Corp. certified stain with dye content 61%): Make 100 mg. % azure A and keep as a stock solution in the refrigerator. Just before use make a 25 mg. % solution from the stock solution. Make *M*/10 borate buffer solution (pH 9.8) and dissolve enough sodium chloride in it to give 0.85%. Use, as standard heparin, crystallin barium salt or sodium salt of heparin. Dissolve unknown sample in physiological saline containing 0.3% tricesol and adjust pH to 9-10 if very strongly buffered. To 2 ml. of buffer-heparin sample add saline to 4 ml. Add 1 ml. of dye solution, mix, transfer to cell of tintometer, and match color quickly, using red and blue glasses. Construct the standard curve by carrying out the procedure with known amounts of standard heparin and plotting the resulting red readings. Dilute unknown solutions until they give a reading in the steep part of the curve, and test further dilutions to show that they result in lower readings.

Heparin isolated as the crystallin barium salt from different species and beef heparin partly inactivated by storage in acid solution at 5°C . for 18 months showed the same metachromatic activity. Since these various sources showed different anticoagulant activity, apparently the measurement of metachromatic activity measures heparin present on a weight basis irrespective of the factors responsible for its anticoagulant activity. If metachromatic measurements are to be made, Lloyd's reagent, often used to purify heparin, must be used with great caution if at all, because it is very strongly metachromatic.—*H. P. Riley.*

LIVINGSTON, R., and PARISER, R. The chlorophyll-sensitized photo-oxidation of phenylhydrazine by methyl red. II. Reactivity of the several forms of methyl red. *J. Amer. Chem. Soc.*, 70, 1510-5. 1948.

Methyl red can exist in three colored forms in methanol, the relative concentration depending upon the acidity of the solution. In the chlorophyll-sensitized photo-oxidation of phenylhydrazine, only the intermediate form of methyl red reacts. The quantum yield is an empirical function of the stoichiometric concentration of phenylhydrazine and of the concentration of the intermediate form of methyl red. Over a fairly wide range the quantum yield is independent of the light intensity and of the concentration of chlorophyll. Further measurements of this type may be of aid to the understanding of the "inner mechanism" of photosynthesis.—*R. T. Whittenberger.*

STAIN TECHNOLOGY

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HEMATOXYLIN-NEUTRAL-RED

A STAIN FOR CELL INCLUSIONS AND CERTAIN TISSUE ELEMENTS

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ABSTRACT.—A method is described for the staining of paraffin sections using hematoxylin as a nuclear stain followed by neutral red. The neutral red is differentiated in aniline-xylene and some viral inclusions and certain cell and tissue elements are brought into prominence by this differentiation and show a specific affinity for the dye.

For a considerable time this laboratory has been engaged in the histological examination of experimental virus material with particular reference to the study of 'inclusion bodies' as an indication of virus infection. From the commencement of the work it was realized that the standard stains for these inclusions were inadequate. Some, such as the Romanowsky modifications demanded very critical differentiation to obtain a distinctive coloration for the inclusions and when these were scanty it was impossible to be certain of a standard result. The 'hot' phloxine methods were time consuming and invariably loosened the section from the slide. By far the most satisfying results were obtained by the phloxine-tartrazine method of Lendrum (1947), but sometimes the differentiation in the tartrazine was very slow and had to be continued for many hours. One of the greatest disadvantages of most of these methods was that an ingested red blood cell could simulate a virus inclusion body if differentiation was incomplete or if it had been mistaken under the staining microscopes for one of these bodies and used as a control.

PROCEDURE

It seemed that a satisfactory stain should have these properties:

- (a) It should be both simple and rapid.
- (b) The nuclear detail must be well shown.
- (c) The counterstain must be of a contrasting color and have an affinity for inclusion bodies but little or no affinity for erythrocytes.

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From past experience it had been realized that the small affinity that neutral red had for erythrocytes could be readily leached away with aniline. It was decided to use this stain following hematoxylin, and the method given below was the one that was tried, and used without modification for all subsequent material.

1. Bring the section down to water.
2. Stain nuclei with Bullard's hematoxylin for 1 minute. (Other hematoxylin may be used).

Bullard's Hematoxylin

50% Alcohol.....	144 ml.	Cool quickly and filter and add:	
Hematoxylin.....	8 g.	95% Alcohol.....	275 ml.
Glacial acetic acid.....	16 ml.	Ammonia alum.....	40 g.
Heat and add:		Glacial acetic acid.....	18 ml.
Distilled water.....	250 ml.	Glycerine.....	330 ml.
Ammonia alum.....	20 g.		
Heat to boiling and add very slowly:			
Mercuric oxide.....	8 g.	Ripen for 1 week—1 month.	

3. Differentiate and "blue" in the usual manner.
4. Stain with a 1% solution of neutral red (Gurr. London, No. 244) in distilled water, for 3 minutes.
5. Rinse in water and differentiate in aniline-xylene (equal parts).
6. Rinse well in xylene to remove the aniline and mount in D.P.X. (polystyrene synthetic resin of Kirkpatrick and Lendrum, 1939).

The differentiation (5) is similar to that used in the Weigert-Gram method, i.e. after rinsing in water to remove the stain at (4) the section is alternately blotted with clean, dry filter paper and flooded with aniline-xylene until the section is 'clear'. When the section is clear it can be left covered with aniline-xylene or immersed in a jar of aniline-xylene until differentiated. It is important that sufficient time be allowed in the differentiator so that the neutral red is adequately leached away. This time is usually between 5 and 10 minutes; at first the nuclei appear as an intense brown-red but as differentiation proceeds they revert back to their original blue-black color of the hematoxylin and the differentiation must always be taken as far as this stage. The fact that aniline-xylene is a clearing agent enables the differentiation to be comfortably appreciated under the high power lens of the staining microscope. Differentiation is stopped by a thorough washing in xylene and the sections should be mounted in one of the synthetic resins, not in balsam. The preparations seem to be permanent.

Most of the tissues examined were fixed in 10% formol saline, but material fixed in formol-sublimate gave comparable results. The stain has chiefly been applied to paraffin embedded material, but

frozen or celloidin sections can be used if the sections are mounted on the slide prior to staining.

As the stain was used it was soon found that although it was excellent for intracytoplasmic viral bodies, no staining of intranuclear bodies were obtained; however, it was soon found that it stained certain cells and tissue elements sharply in shades of rose-pink to brown-red, and has proved a valuable general stain. The following table sets out the results so far obtained.

Granules or Cytoplasmic Bodies in Normal Cells

- Positive** Cytoplasmic granules in eosinophils, bright red. Cytoplasmic granules in basophils (tissue mast cells), dull red. Basophil cells in pars anterior and pars intermedia of pituitary, red brown.
- Negative** Oxyphil granules in Paneth cells and of oxyntic (parietal cells) of stomach; zymogen granules of pancreas and salivary gland cells, granules of islet cells of the pancreas, granules of enterchromaffin cells, granules (except basophil) of pituitary, granules of neutrophil leucocytes. Nissl bodies (tigroid) of ganglion cells.

Cytoplasmic Bodies in Abnormal Cells

- Positive** Russell's bodies in plasma cells, cytoplasmic bodies in Hofbauer cells, Foa Kurloff bodies in the lymphocytes of guinea pig, all bright red. Corpora amylacea in neuroglia, red violet.

Inclusion Bodies and Virus Particles

- Positive** Molluscum contagiosum, old rose. Giant cell pneumonia of infancy, old rose. Cowpox (chorioallantoic membrane), bright red. Variola (chorioallantoic membrane), faint pink. Ectromelia (chorioallantoic membrane), bright red to a pale pink. Rabies (intracytoplasmic Negri bodies), red. Psittacosis (elementary bodies and morulae on chorioallantoic membrane), dark brown. Typhus rickettsiae (Nigg and Brioul strain in rat lung), red brown, and granular bodies bright red.
- Negative** Generalized vaccinia (intracytoplasmic and intranuclear). Inclusion encephalitis (intranuclear). Herpes in brain (intranuclear).

Other Parasites and Micro-organisms

- Positive** Gram positive and negative organisms and fungi, dark red brown. Kala azar (Leishman-Donovan bodies), red purple. Torula (mucopolysaccharide coating), red brown. Histoplasma (capsule of yeast forms), light red.
- Negative** Acid fast bacilli (tubercle, leprosy including globi).

Other Tissue Elements

- Positive** Connective tissue mucin, brown red. Thyroid colloid, bright red. Exudate with high protein content, red. Fibrinoid red. Amyloid (infective), rose pink.
- Head of spermatozoa, brown red. Cartilaginous stroma, orange red. Keratin, bright red. Charcot-Leyden crystals, bright red.
- Negative** Amyloid (primary systemic), fibrin, erythrocytes.

On occasions prominent nucleoli retain the neutral red and this is particularly marked in the chorioallantoic membrane and they should not be mistaken for intranuclear inclusion bodies; under certain conditions which have not been precisely defined, but are probably associated with imperfect fixation, a certain proportion of the nuclei of lymphocytes are stained red. Some substances such as gastric mucin are very retentive of the dye.

DISCUSSION

Neutral red—(C. I. 825) the chloride of toluylene red, is a weakly basic amido-azin dye and was discovered by Witt in 1879. It was introduced into microscopy by Ehrlich (1894) as an intravital and supravital dye, and this, together with its properties as an indicator has been its principle application in histology up to date. Unna and Golodetz (1917) prepared a mixture of two parts of neutral red and one part of new blue which they called "Neutral Violet Extra" and used it for the study of oxidation-reduction potentials in frozen sections of unfixed material. Rosin (1894) found that a concentrated watery solution of the dye was the most effective method of displaying Nissl granules in formalin-fixed material and Schmidtman (1916) recommended neutral red and picric acid for the fine structure of striated muscle fibers. Twort (1924) introduced a neutral stain for parasites in tissues, dissolving in weak alcohol the precipitate formed on mixing aqueous solutions of neutral red and light green. Faris (1924) used neutral red and Janus green as a general tissue stain for fixed embryological material but found it difficult to differentiate overstained sections.

However, so far as it is known, hematoxylin and neutral red have not been used in combination, partly perhaps because the use of two nuclear stains in succession seems irrational and partly perhaps because neutral red has been regarded as an 'end-point' not capable of specific differentiation. The chemical principles underlying the specificity of neutral red when used in this way are being investigated. Mann (1902) following Heidenhain's (1902) theory suggests that neutral red and neutral violet, both being weakly basic dyes, would react in an analogous manner to proteins, but it has been found that if neutral violet is substituted for neutral red, there is no specific staining. It seems more likely that in some way neutral red is functioning as an indicator of differences in the acid and base combining properties of the proteins in the fixed tissues for there is good correlation between those structures regarded by Unna (1928) as being formed of oxidizing proteins and the tissue elements stained by neutral red by this method.

ACKNOWLEDGEMENTS

I would like to thank Dr. Robb-Smith for his direction and constant encouragement in this study. I would also like to thank various pathologists who kindly supplied some of the material examined.

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NEGATIVE-STAINING OF PROTOZOA WITH NIGROSIN

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ABSTRACT.—The use of nigrosin in a rapid but accurate method for the demonstration of cilia bands, vacuoles and cytoplasmic inclusions in infusorians is discussed. The general effects of the dye as well as its staining action are presented together with a brief explanation of the physics of the action. The technic proposed involves mixing a drop of infusion on the slide with a drop of 10% aqueous nigrosin; excess stain is drained off, the specimen dried and mounted.

INTRODUCTION

Although early workers regarded nigrosin as a favorable agent for the staining of nervous tissue, the dye since then has found use in the staining of fungi spores and algae (Dorner, 1922). The peculiar properties of nigrosin also make it admirably suited for the demonstration of certain structures of infusorians which are otherwise difficult to stain.

All workers have reported nigrosin as a strong decolorizing agent, even noting that as high a dilution as 1:100 has this effect (Dutton, 1928). Its toxic effects are mentioned by Monné (1935), but when used as a vital stain it is not utilized to the best of its advantages. As a capsule stain in bacteriology, it has marked advantages. Dorner (1930), Cumley (1935) and Fleming (1941) all have reported negative-staining of bacteria with enthusiasm. It produces rapid and accurate staining and further, overstaining cannot occur. Finally, since high temperatures need not be used in fixing specimens, subsequent distortion of the cell through heat does not occur.

It will suffice here to outline the ramifications of negative-staining with particular reference to nigrosin in the staining of protozoa and certain of their external structures.

PRINCIPLES OF THE TECHNIC

While it is generally agreed that any staining process involves both physical and chemical factors, the physical phenomena which occur during negative-staining are of particular interest. While the exact chemical composition of nigrosin is not known, it consists of a

mixture of indulin and a yellow component resulting in a compound with a color range from black to violet. It has an absorption ratio of $575 \text{ m}\mu/605 \text{ m}\mu = 1.01$. Peterson, Conn, and Melin (1934) state that nigrosin is produced by the sulfonation of the products resulting from the interaction of anilin, anilin hydrochloride and nitrophenol.

Essentially, negative-staining consists of staining the background of the field of observation, leaving the actual specimen unstained. Later, if desired, the specimen can be differentially stained. Maneval (1934) and Cumley (1935) have reported obtaining good results with such interesting dyes as China blue, Poirier's blue and Congo red. Other workers have employed combinations with orange G, eosin, carmine and cotton-blue. The results in all cases have resulted in striking, although not altogether permanent, preparations.

Nigrosin probably depends upon colloidal reactions in its staining



FIGURE 1

A and B. Schematic representation of the effect produced when the stain film is too thick (B). A is an ideal case.

C. Illustrating the adhesive effect of nigrosin in staining cilia. In both drawings nigrosin is represented by intense black.

effects. Along these lines, Burri (1909) mentions india ink as ideally suited for negative-staining. India ink produces an opaque background film of suitable thickness for the process. Nigrosin, on the other hand, has the ability to disperse more freely in a liquid phase, and hence, the resulting suspension is more fluid than india ink

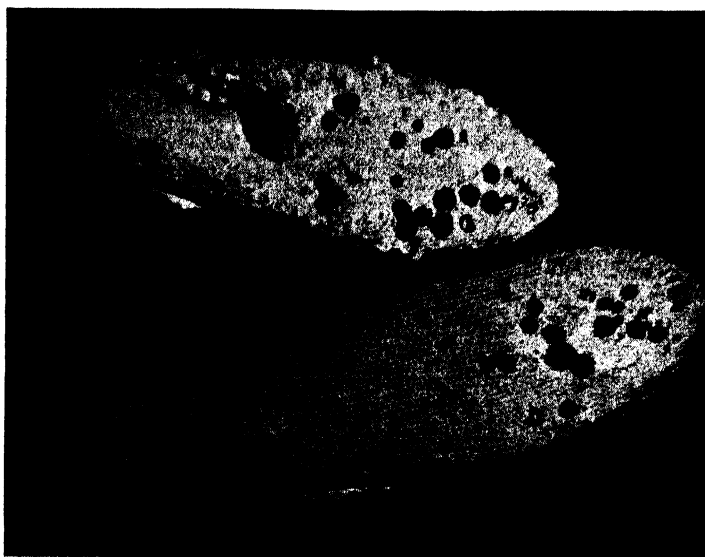
PLATE ONE

Fig. 2 (Top).

Negative stain preparation of *Paramecium multimicronucleatum*. $\times 450$. Note the demonstration of the processes of cilia and the cytoplasmic inclusions at the anterior end.

Fig. 3 (Bottom).

Nigrosin-stained *Paramecium multimicronucleatum*. $\times 150$. There is a slight accumulation of the dye about the outer edges of the specimens but, in general, distortion is brought down to a minimum.



suspensions of the same density. Ideally, the stain film produced by any background stain should be one-half the thickness of the specimen concerned. While no harm occurs when the film is less than one-half the thickness of the specimen, the external structures are not so easily defined. It is needless to say that films of greater than one-half thickness will give a distorted view of any specimen. (See Fig. 1).

The dye, when nigrosin is used as a background stain, appears to adhere to bodies in such a manner that, with a free flowing solution, a very thin film stains very well without completely covering the specimen. With large specimens (particularly with certain of the large amebae), dark pockets of the stain may form about the hyaline layer of the specimen; this is apparently a result of the intense adhesive quality of the staining solution and may be prevented by the technic to be outlined presently.

A problem occurs when one is to make selection of a fixing agent. Since one of the advantages of this technic is the elimination of distortion, high temperatures should be avoided. Many workers have employed picric acid as a fixing agent, occasionally mixing it with nigrosin directly. In the latter state, however, the picric acid was found to act as a stain which later faded out. The other alternative was to wash the acid out, which took considerable time and effort.

Of the other standard fixatives, Bouin's produced unsatisfactory results while nearly all the sublimate formulae worked quite well. The use of the stain itself as a fixing agent proved to be the most satisfactory method of fixation. Since washing of the slide to remove the stain is unnecessary, several steps are accomplished in one.

One of the interesting results of staining with nigrosin by this method is its demonstration of the cilia pattern of infusoria (see Fig. 2). The results are probably the result of the capillary effect previously described. The specimen is first suspended in the staining solution until the film is spread out on the slide. As the stain is dispersed out, the upper half of the specimen breaks away from the stain with small portions of the dye left clinging to the cilia by capillarity (see Fig. 2 and 3).

During the course of work carried out in the summer of 1946, the writer had occasion to note the effect of certain dye suspensions on *Pelomyxa*. Curiously enough, nigrosin appeared to "take" and hence the central body surface of this amoeboid form was stained merely a faint purple against an almost black back-ground. The internal morphology was not visible when compared to the results obtained by Burri's india ink technic and the outer protoplasm did not take at all. This suggests immediately that homogeneous par-

ticles of nigrosin form, in their dispersed phase, close contact with each other on body surfaces and in solution so as to produce a high surface tension with great capillarity. Also, the cuticle of infusorians offers a splendid surface for the action of nigrosin, while the outer viscous hyaline zone of modified protoplasm found in *Pelomyxa* offers no satisfactory surface for the differential back-ground stain, since only the central granular substance was stained.

Deflanche (1929) and Anselmier (1930) have employed nigrosin in the study of infusorians, describing its use with a mordant and fixation with Caesar's-Gil's fluid. The present method herein described presents a rapid technic for the demonstration of ciliary bands, basal bodies and cytoplasmic inclusions in both temporary smears and permanent mounts, without use of mordants or complicated fixation.

TECHNIC

The dye is utilized best in aqueous concentrations of 1:10. Ten grams of nigrosin are boiled in 100 ml. of distilled water for about 30 minutes. To remove undissolved material, the solution should be filtered several times and a few drops of formalin added to prevent decomposition. All slides must be free from dust and grease similar to slides used for blood smears. The recommended procedure is as follows:

1. One drop of animal-containing infusion is put on the slide and allowed to spread of its own accord.

2. A drop of 10% aqueous nigrosin is then put on this drop and the whole mixed and spread on the slide.

3. In order to obtain the satisfactory thickness of the staining film about the specimen, the slide should now be put on its side and a piece of blotting paper held against the edge. In this way, the excess stain is drained off and, at the same time, the specimens are thoroughly exposed to the dye without formation of dark stain pockets. No loss of specimens was noted during this process.

4. The slide is now ready to be put on a warming table where, after the film has dried, it may be examined and mountant and cover-slip applied. Clarite makes an ideal mountant. No discoloration has as yet (after 2 years) been noted.

Results are as follows: Ciliary bands—black against the transparent cuticle. Nucleus—faint purple to purple-black. Vacuoles and basal bodies—black.

The author wishes to express his thanks to Dr. J. N. Dent of the Department of Biological Sciences of the University of Pittsburgh for his criticisms of this note and to Dr. Ernest L. Hunt for his assistance on the photomicrographs.

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DIFFERENTIATING YEAST ASCOSPORES AND VEGETATIVE CELLS

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ABSTRACT.—A procedure is described for staining ascosporeogenous yeasts with crystal violet, acid alcohol, and safranin. The spores are stained violet, and the vegetative cells red.

As a rule, ascospores of yeasts are acid-fast and possess staining properties similar to those of bacterial endospores. Among acid-fast stains which have been suggested for sporulating yeasts is the method of Beauverie (1917) in which the ascospores are stained with carbol-fuchsin and vegetative cells with thionin. Shimwell (1938) employed malachite green and safranin for a differential stain in which the ascospores appeared green and the vegetative cells red. Two technics were devised by Ochmann (1929) in which the primary and counterstains were, respectively, methylene blue with Bismarck brown, and fuchsin with gentian violet.

The method described here is a modification of previous acid-fast methods. Crystal violet is applied for the primary stain followed by decolorization with acid-alcohol and subsequent counterstaining with safranin.

It is suggested that this technic should be of value in the small laboratory where staining reagents other than those used in the Gram stain are not routinely employed.

PROCEDURE

A mixture of yeast ascospores and vegetative cells was obtained by growing *Saccharomyces cerevisiae*² on carrot slices for one week. Slides were smeared with distilled water suspensions of this growth and stained by the following procedure:

1. Allow film to air dry, then heat-fix lightly.

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²This staining procedure has also given good results with other species of sporulating yeasts.

2. Flood slide with aniline crystal violet and heat gently for three minutes, replenishing the stain as it evaporates. The slide should be heated to the point where steam is given off, but the stain should not be allowed to boil.

Composition of aniline crystal violet

Crystal violet ³	5 g.
Ethyl alcohol (95%)	10 ml.
Aniline.....	2 ml.
Distilled water.....	20 ml.

3. Rinse slide in tap water and destain for 15 seconds with 95% ethyl alcohol containing 3% HCl.
4. Rinse off acid-alcohol with tap water and stain lightly with safranin (10 to 15 seconds).

Composition of safranin

Alcoholic safranin (2.5% safranin O ⁴ in 95% alcohol).....	10 ml.
Distilled water.....	100 ml.

5. Rinse in tap water, blot dry.

In this method, the spores are stained deep violet as contrasted with light pink vegetative cells. Too long an application of safranin will cause the vegetative cells to appear darker and lessen the differentiation.

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³Crystal violet, National Aniline Division, Allied Chemical and Dye Corp., Certification No. NC 32, dye content 92%.

⁴Safranin O, National Aniline Division, Allied Chemical and Dye Corp., Certification No. NS 19, dye content 86%.

A RAPID ROSIN-CELLOIDIN-PARAFFIN METHOD FOR EMBEDDING TISSUES

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ABSTRACT.—Pieces of tissue of various sizes or tissue fragments are dehydrated in 95% alcohol, cleared, washed with ether and infiltrated with a solution containing parloidin 9.0 g., camphor 3.0 g., absolute alcohol 200.0 ml., ether 200.0 ml., rosin 45.0 g. and castor oil 10 drops. After evaporation to the desired consistency, the mass is hardened with chloroform vapor, trimmed, passed through 3 changes of xylene to remove the rosin, embedded in paraffin, sectioned serially, stretched and stained as for paraffin methods. Methods of defatting tissues and detailed procedure for embedding fragments of bone marrow by this method are given.

The celloidin procedure here described is the result of a series of attempts, beginning in 1938, to obtain a rapid, double-embedding medium that would meet requirements for cutting serial cross-sections of lymphoid nodules in the appendix of the rabbit which when sectioned in paraffin lose some of the free lympho-epithelial apices (Crabb and Kelsall, 1940, Fig. 1, levels F and T; 5 and 7). Late in 1947, after many modifications had been tried, the present formula and procedure were adopted and have proved to be satisfactory for routine work with fragments and blocks of tissues.

Early in the investigation the difficulties encountered with the various celloidin combinations indicated that a desirable embedding medium should meet the following requirements: (a) the solution should penetrate tissues readily, (b) the hardened celloidin should be sufficiently porous to permit ready infiltration by paraffin, (c) it should not wrinkle from the heat used in stretching the ribbons, and (d) it should be chromophobic.

Previous experiments with rosin in formulae for a binder to be used in connection with military explosives and as a hardening agent in waxes for embedding tissues suggested that rosin could be incorporated in a celloidin medium and, after the tissue had been embedded in

¹This article resulted from work in progress which is supported by research grants from the Beta Sigma Phi International Endowment Fund and the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

this medium, dissolved out to facilitate impregnation of the celloidin block with paraffin. Increasing the amount of rosin reduced the amount of time necessary to infiltrate the block with paraffin, but excess rosin resulted in a very granular or retiform background which was objectionable for certain work, especially when high magnification was used. Thus, it was found desirable to adapt the amount of rosin used to the character of the tissue and nature of the investigation.

The use of collodion flexible (U. S. P., General Chemical Co., Lot No. 18) sufficiently reduced wrinkling in stretching the ribbons to suggest that a flexible celloidin should be used. A series of combinations that included either or both Collodion flexible and Parloidin (Pyroxylin purified, Mallinckrodt) as the basic ingredient with amyl acetate (purified, J. T. Baker, Lot No. 52230), clove oil and other solvents were tried, but none was satisfactory. The idea of using camphor and castor oil instead of the more volatile amyl acetate was derived from two pharmacopeias (Remington, 1905, *Practice of Pharmacy*, 4th Ed., p. 320; Sollmann, 1932, *A Manual of Pharmacology*, 4th Ed., p. 145); but the quantity of these ingredients had to be determined by trial.

This method is well adapted to cutting serial sections of small organisms, certain ova, tissue fragments of various kinds and fairly thick pieces of a wide range of structures that ordinarily should be sectioned by the more laborious celloidin method. It is especially useful in sectioning organs that are likely to lose minute parts and difficult structures, such as cross-sections of the limbs of small mammals that include skin and decalcified bone. Obviously, this method is not adapted to work with cell inclusions or tissues that would be injured by the temperature of the paraffin bath. Since the size of the block of tissue and other factors increase the minimum length of time necessary for the various steps in infiltrating larger pieces, the only detailed procedure given is for tissue fragments, from which the investigator will be able to formulate a schedule to meet his needs.

The problem of serially sectioning fragments of fixed bone marrow of hamsters was solved satisfactorily and more rapidly by this procedure than by any of the paraffin (Crabb, 1927; O'Brien, 1947) or double-embedding (Bensley and Bensley, 1938; Gatenby and Cowdry, 1928; and Lillie, 1948) methods tried. Fragments of marrow from femora and humeri that had been cracked before fixation were put into 10 ml. shell vials containing 70% alcohol, to which a drop of weak aqueous solution of acid fuchsin had been added to make the fragments readily visible, passed, by decanting, through three changes of 95% alcohol, followed by three changes of ether (anhy-

drous is preferred) or by carboxylol or anilin. Either of the two clearing agents should be removed with ether. The 95% alcohol may be followed by absolute alcohol instead of ether or a clearing solution but, due to atmospheric moisture, the results obtained are not as constant as when a clearing agent followed by ether is used.

The rosin-celloidin solution, here denoted as "Pi", is poured into the vial and the fragments of marrow arranged in the form of a rectangular heap, by using a dissecting needle that has been bent to a right angle near the tip and ground flat on two sides. The fragments may be collected by centrifugal force after the method of O'Brien (1947); however, use of the bent dissecting needle is efficient and more rapid. After the fragments have been arranged in the Pi solution, the mass is allowed to evaporate to a thick jel, or to any desired consistency, and solidified with chloroform vapor from a saturated pledget of cotton retained by the stopper. After hardening, the excess mass is trimmed away and the solid block containing the tissue fragments is placed into xylene to remove the rosin in order to leave the celloidin matrix porous and thus facilitate impregnation with paraffin. The block is then infiltrated and embedded in paraffin of a suitable melting point and sectioned with a rotary microtome.

The judicious use of heat (40-60°C.) will hasten any of the steps in defatting, clearing and infiltrating tissue, but may result in expelled stoppers and desiccated tissue. Koneff and Lyons (1937) recommend use of screw-topped jars heated in an oven, but loosely stoppered shell vials under a desk light are used in this laboratory.

Although the ingredients of the Pi solution and the methods of using it may be modified to suit a number of needs, the following formula gives good results for routinely embedding both fragments and larger pieces of tissue:

Parloldin (Pyroxylin Purif., Mallinckrodt) dry	9.0 g.
Camphor, refined "gum" or USP, Dupont	3.0 g.
Ethanol, absolute	200.0 ml.
Ether (anhydrous is preferred)	200.0 ml.
(Dissolve parloldin before adding rosin)	
Rosin, selected, clear lump, pulverized	45.0 g.
Castor oil (oleum ricini)	10 drops

It requires 24 to 48 hours at room temperature, with occasional shaking, for the parloldin to dissolve and another 5-24 hours for the rosin to go into solution. Some samples of commercial natural refined camphor "gum" seem to give the Pi mass more lasting flexibility than synthetic camphor. Since the solution keeps well in the dark a stock may be kept on hand indefinitely.

The Pi solution penetrates tissues more rapidly than the commonly

used solutions of nitrocellulose, and after the block has been hardened it remains flexible for months. For example, a cylindrical piece of uninfiltrated Pi mass, originally 10×20 mm. and very soft, that was exposed to room atmosphere more than two years, shrunk about 50% but did not become too hard to section. After the rosin has been removed and the mass thoroughly infiltrated with paraffin no further shrinkage occurs, and blocks thus treated and stored for two years sectioned readily.

The following procedure is well adapted for fragments of fixed bone marrow in 70% alcohol when 10 ml. shell vials are used and the fluid is changed by decanting. If larger pieces of tissue are to be infiltrated, the volume of liquid and the time for each step should be increased in proportion to the size of the pieces.

PROCEDURE FOR BONE MARROW

Fragments in 70% alcohol	Minutes
1. 95% alcohol.....	10
2. 95% alcohol.....	5
3. Carboxylol.....	10
4. Ether, 3 changes, total time.....	10
5. Pi solution (may remain several days).....	30
6. Arrange fragments in vial and evaporate to a firm gel or solid (1-12 hr.).....	90
7. Chloroform vapor (1-12 hr.).....	30
8. Trim and clear in carboxylol or anilin.....	15
9. Xylene, 3 changes, total time.....	15
10. Infiltrate in paraffin (1-5 hr.).....	60
11. Embed, section with rotary microtome.....	
12. Stretch ribbons as for paraffin sections.....	

Although the steps in this procedure may be completed in relatively short time, most tissues are not injured by greatly prolonging the time. Thus, the great flexibility in choice of fixing, dehydrating and clearing agents and in adapting the schedule to suit the convenience of the investigator make this a very useful method for serially sectioning a wide variety of difficult tissues.

VARYING THE PROCEDURE

If it is desired to modify the Pi formula or the procedure, one should bear in mind that this is an anhydrous solution and that rosin is soluble in alcohol, ether, chloroform, xylene, and probably in a number of other substances used in microtechnic. Although the tissue in a block of Pi that has been hardened in chloroform may be further hardened in the air without injury, the Pi-embedded tissue should never be exposed to appreciable desiccation after the rosin has been removed and before the tissue is infiltrated with paraffin.

DEFATTING VARIOUS TISSUES

Tissue that contains fat or oil should be defatted before an attempt is made to infiltrate it with either Pi solution or paraffin. Appreciable traces of fat in tissues may be removed by following the carboxylol (step 3) with xylene and subjecting the tissue to a temperature of 35 to 60°C. for 15 to 60 minutes. The xylene should be changed two or three times and removed with ether before the tissue is passed into the Pi solution. Excessive fat in larger pieces of tissue may be removed by using two or more changes of the following solution after step 3 for several minutes at 35 to 50°C. or for several days at room temperature.

Fat Solvent

Ether	40.0 ml.
Chloroform	40.0 ml.
Oil of turpentine, rectified	20.0 ml.
Sudan III (optional)	trace

All traces of chloroform and turpentine should be removed with ether before the tissue is subjected to the Pi solution (step 5). Students and assistants should be warned that the ether used in this method is highly inflammable and may be dangerously explosive.

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STAINING THE TUBERCLE ORGANISM IN SPUTUM SMEARS

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Technicians with whom the writer has been associated have objected to the unmodified Ziehl-Neelsen technic for staining tubercle organisms in smears because of the messy precipitates obtained when the staining fluid is heated on the slide. They claim that more brilliant staining, as well as cleaner-looking slides, can be obtained by the Kinyoun (1915) method, which employs a stronger carbol-fuchsin solution with cold staining in a Coplin jar.

This claim seemed worth investigation, and preliminary tests indicated its correctness. At the same time it became apparent that the Kinyoun solution is illogical, in that it calls for an over-saturated (8%) solution of phenol and a 3.3% solution of basic fuchsin in about 17% alcohol (which would probably be impossible except with pararosanilin acetate, the most soluble of the primary constituents of basic fuchsin). Accordingly it seemed worth while to determine whether equally good results could be obtained by cold application of a less concentrated carbol fuchsin solution.

In this investigation, the Kinyoun solution (filtered to remove excess dye and phenol), the usual Ziehl-Neelsen formula (0.3% basic fuchsin and 5% phenol in about 10% alcohol), and a third formula intermediate in strength between these two, were employed. No appreciable difference between the three solutions could be observed; in every case where the stain was applied cold, the results were fully as sharp, and the slides much cleaner than when the fluid was heated on the slide as by the conventional technic. No longer staining than called for by the ordinary Ziehl-Neelsen technic (3-5 minutes) was required. In some cases it proved desirable to reduce slightly the decolorizing period; decolorization for 2 minutes in acid alcohol was in every case sufficient.

Accordingly it seems unnecessary to employ heat when staining smears for acid-fast staining, or to modify the regular Ziehl-Neelsen formula when the stain is applied cold. Cold staining is simpler than the usual method, and is if anything quicker, when staining jars are employed, as several slides can be immersed at once, and

close watching is unnecessary. The technic is simply: 3-5 minutes in the cold staining fluid; rinsing in tap water; 15 seconds to 2 minutes in acid alcohol (3% HCl, by volume, in 70 to 95% ethyl alcohol); washing in tap water; 1 minute in 1% aqueous (or Loeffler's) methylene blue; washing in tap water. In case of a negative result, it is always possible to check the findings by the conventional hot-staining method, but the writer has so far found this unnecessary.

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HYDROCHLORIC ACID AS A FIXATIVE FOR ROOT TIP CHROMOSOMES

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ABSTRACT.—After pretreatment with 0.2 to 0.3% colchicine at 26° C. for 2 hours root tips are fixed and macerated in a 1/10 dilution of concentrated hydrochloric acid at 60° C. for 10 to 14 minutes, washed, transferred to a staining dish with aceto-orcein or another aceto-stain for about 10 minutes, immersed in a drop of the stain, covered and squashed. The preparations may subsequently be made permanent.

Hydrochloric acid has been used heretofore as a macerating agent following fixation of plant tissues with alcohol-acetic-acid or alcohol-chloroform-acetic-acid combination (cf. Darlington and La Cour, 1942). In this laboratory fine results were obtained by omitting these fixatives, and instead, fixing live root tips directly in hydrochloric acid, combining fixation and macerating hydrolysis into one step. Considerable time and material is thus saved, and metaphase preparations are obtained which equal in every respect those prepared with the older fixatives. The chromosomes stain as intensely (perhaps even more so) with this technic as after fixation in 3 parts absolute alcohol to 1 part glacial acetic acid. The new schedule was used mainly for a study of the karyotype of the composite *Scorzonera tau-saghyz* Lipsch. et Bosse, a rubber bearing plant of Russian origin. Good results were also obtained with root tips of guayule (*Parthenium argentatum*), (pretreated with paradichlorobenzene; v. Meyer, 1945), tobacco and tomato. The schedule for *Scorzonera tau-saghyz* follows:

1. *Pretreatment*: Seedlings germinated on moist filter paper are immersed in colchicine to effect a spreading of the chromosomes (O'Mara, 1939). Pretreatment with paradichlorobenzene (Meyer, 1945) has failed to give distinct constrictions, and pretreatment with cold (Delaunay, 1930) which was effective at 5°C., has reduced considerably the number of metaphases. The temperature for the colchicine treatment should be 26°C. and last for two hours. Concentrations used by the author have ranged from 0.2 to 0.3% (aqueous), the former concentration giving little, and the latter extreme shortening

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of the metaphase chromosomes. Following pretreatment, the seedlings are rinsed in distilled water.

2. *Combined fixation and hydrolysis:* Seedlings are transferred to a 1:10 aqueous dilution of hydrochloric acid at 60°C. for 10-14 minutes. Then they are rinsed thoroughly to stop hydrolysis.

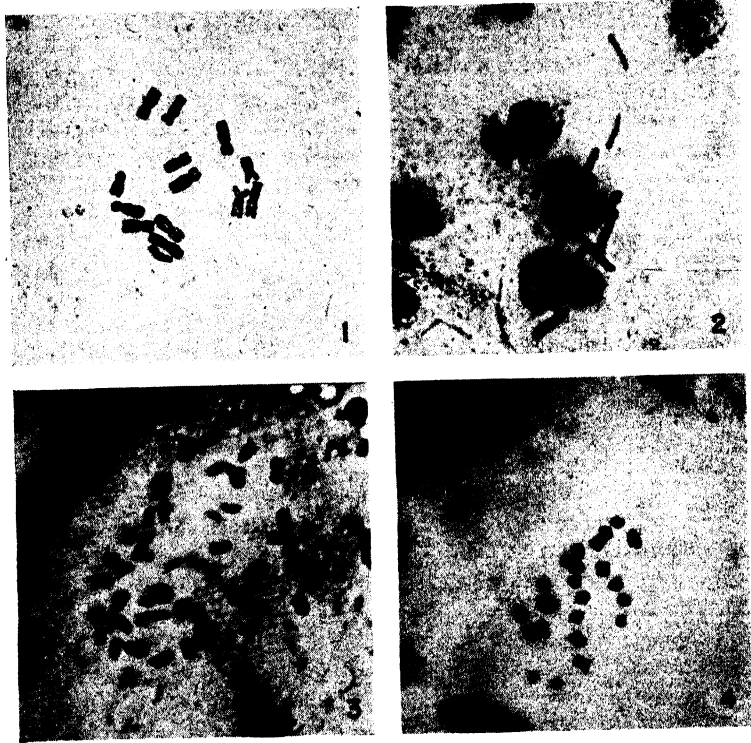


Fig. 1-4. Root tip chromosomes fixed in hydrochloric acid. Fig. 1-2. Tausaghyz, $\times 900$. Fig. 3. Guayule, $\times 1800$. Fig. 4. Tomato, $\times 1800$. Fig. 1, 2, and 4 are pretreated with colchicine, Fig. 3 with paradichlorobenzene. The chromosomes in Fig. 1 and 4 are highly contracted by the pretreatment and those in Fig. 2 and 3 are little affected. Fig. 1 is stained with aceto-carmine, Fig. 2-4 with aceto-orcein.

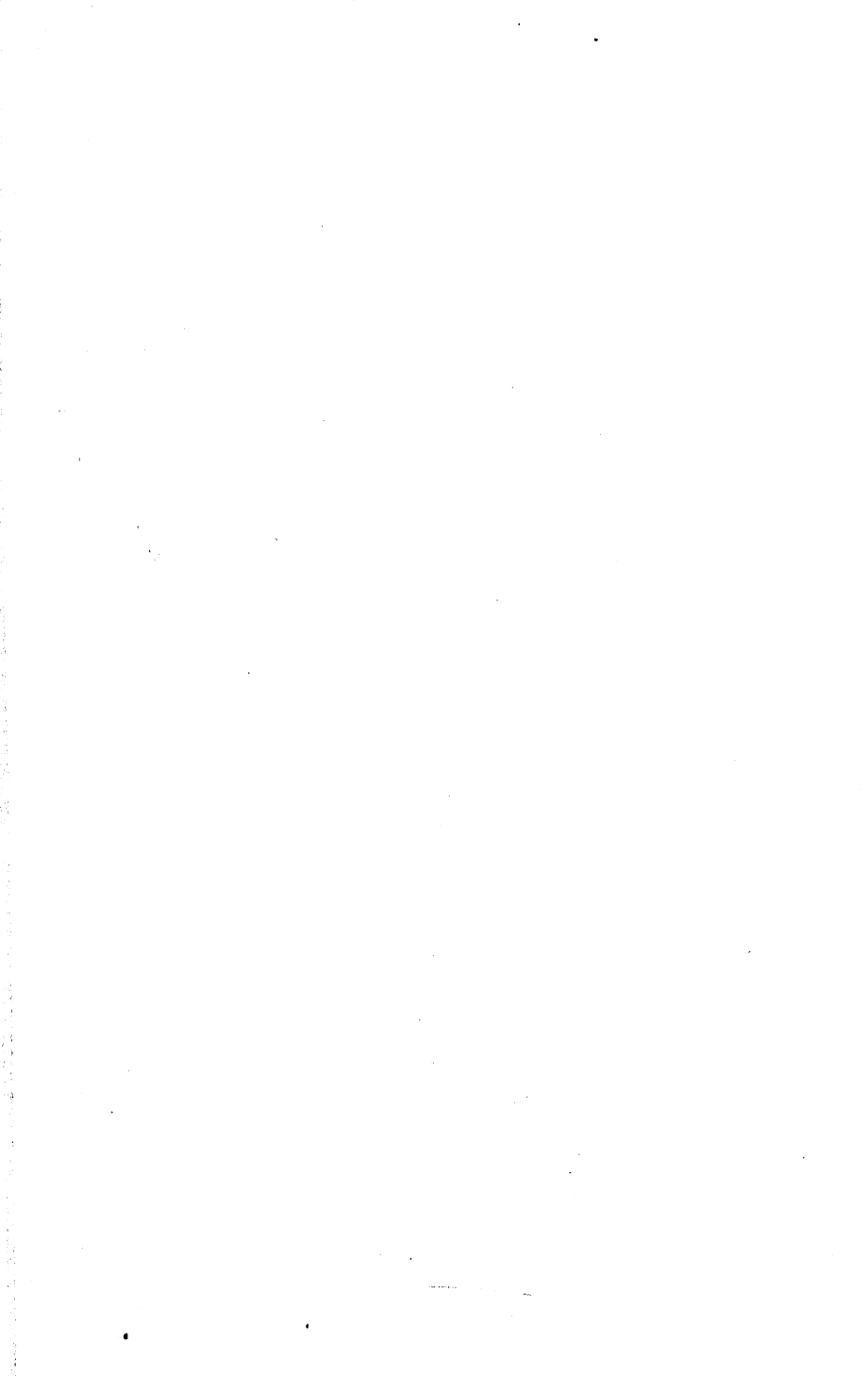
3. *Staining:* Seedlings are transferred in bulk to a staining dish containing aceto-orcein and after about 10 minutes placed singly on slides in a drop of the stain, where all tissues but the root tips are cut off with a spear needle and discarded. Instead of orcein, aceto-carmine or aceto-lacmoid (La Cour, 1947) may be used as stains.

4. *Covering and sealing*: The preparations are covered with cover slips and the cells are spread by tapping with a wood handle or by pressure upon the cover slip and sealed with a paraffin-mastic gum mixture.

5. The preparation may be made permanent, immediately or after several weeks, by the methods of Tobgy (1942) or Meyer (1945), consisting in floating off the cover slips in acetic alcohol, transferring cover and slide to absolute alcohol and recombining them in diaphane (or cedarwood oil for aceto-lacmoid).

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A METHOD FOR MASS PREPARATION OF MULTIPLE HISTOPATHOLOGICAL SLIDES

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ABSTRACT.—A method for the rapid preparation and staining of multiple mammalian tissues is described. With this method sections from 8-10 different organs can be handled simultaneously, mounted on the same slide, and stained with equally good differentiation and color contrast. It gives good results with either Zenker's or formalin-alcohol fixation.

The method to be described has enabled the author to prepare within a few days the same number of slides that, with ordinary techniques, formerly required several weeks' work. With this method, slides from 50-100 test animals (dogs, rabbits, guinea pigs, and rats) involving about 500-1000 tissue sections have been prepared within a week. In addition to saving time, the procedure is convenient and inexpensive, and eliminates exposure of personnel to the fumes of toxic dehydrating and clearing agents.

Routine fixation is used: pieces from each animal are bundled individually in 60-gauge cheesecloth, fixed in Zenker's or formalin (half 10% formalin, half 5% alcohol), washed and brought up to 70% alcohol.

Dehydration, Clearing, Infiltration and Embedding¹

1. Isopropyl alcohol 70% 1 hour
2. Isopropyl alcohol 99% 1 hour
3. Isopropyl alcohol 99% 2 hours
4. Isopropyl alcohol 99% (at 45°-52°C.) 3 hours
5. Melted paraffin (pure, clean and of fine quality, melting point 54.4-56.1°C.) with bayberry wax . 1 hour.

Note: Filter hot paraffin with bayberry wax; 10 pounds paraffin, 40 grams bayberry wax.

6. Fresh melted paraffin with bayberry wax—overnight.
7. Fresh melted paraffin with bayberry wax—all the following morning.

¹Modification of the method described by Duxtader, Elton K.: Isopropyl alcohol in the paraffin infiltration technic, *Stain Techn.*, 23, 1-2, 1948.

8. Embed and block in fresh melted paraffin with bayberry wax—in afternoon. Paper boxes $1 \times 1 \times 15$ inches are most convenient, as the tag number can be written on each. As many as ten pieces can be placed in one box.

Embedding Technic: Place the sections from each test animal in individual Stender dishes containing melted paraffin, placing the tag number with the pieces. Keep the dishes in a paraffin bath set at 56° – 60° C.

Pour hot melted paraffin into the boxes until they are nearly filled. Place the tissues into boxes in an order which will enable the type of cutting desired, e.g. for cross-sections. If necessary add more paraffin, until the tissues are well covered. Set the blocks aside until the surfaces solidify; then plunge the entire block into ice water. When completely hardened, drain the blocks by placing them upside down on paper towels.

The paraffin block from each test animal should be mounted for sectioning, placed in an individual beaker of ice water with its tag number, and stored in the ice box until ready for cutting. Sections are cut 5 – 7μ thick with the microtome.

*Staining:*² Thirty to 50 slides can be stained simultaneously with staining dishes No. 9204 (Arthur H. Thomas). The slides are placed horizontally, each in an individual slot.

1. Xylene..... 3 minutes
2. Xylene..... 2 minutes
3. Iodine Solution..... 10 minutes
(Tincture of iodine added to 70% alcohol until solution acquires a deep port-wine color).

Check under the microscope to make sure that all traces of the fixative have been removed.

4. Ethyl alcohol, 95%..... 5 minutes
5. For bleaching, if any trace of iodine remains, place slide in 5% aqueous solution of sodium hyposulphite until no yellow or brownish color remains; then wash in distilled water.
6. Harris alum Hematoxylin full strength..... 8 minutes
 - (a) 1 gram hematoxylin in 10 ml. absolute alcohol.
 - (b) 20 grams ammonium or potassium alum in 200 ml. distilled water, dissolved with the aid of heat.

²Modification of the method described by Rosenbaum, Robert: Phloxin as an histological stain, *Stain Techn.*, 22, 149–52, 1947.

- (c) Mix (1) and (2) and add 0.5 gram mercuric oxide and bring quickly to boiling. Cool in cold water.
- (d) Mallory recommends adding 5% of acetic acid.
7. Tap water. 5 minutes
(Distilled water made slightly alkaline by the addition of a little sodium carbonate or a few drops of saturated aqueous solution of lithium carbonate may be substituted to bring out the blue-black of the hematoxylin.)
 8. Acid alcohol (0.5% concentrated HCl in 70% alcohol)—30 to 60 seconds. (Control differentiation by keeping rack with sections in constant motion).
 9. Wash with tap water.
 10. Scotts bluer (KHCO_3 , 2 grams; MgSO_4 , 20 grams; distilled water to make 1000 ml. of solution). 2 minutes
 11. Distilled water rinse.
 12. Phloxine (Mallory). 5 to 10 seconds
 13. Alcohol 95%. 5 minutes
 14. Absolute alcohol. 5 minutes
 15. Xylene. 5 minutes
 16. Mount in resin mounting medium in toluene solution ('Harleco', Hartman Leddson Company, 58th & Market Streets, Philadelphia, Pa.).

The slides are clear with excellent differentiation and contrast. The nuclei are bluish-purple, the background purplish-pink. This method has been used with sections of liver, spleen, kidney, heart, stomach, intestine, lung, adrenal glands, ganglia, brain and cords, mounted on the same slide. Results are equally good with all tissues.

The author is indebted to Mary Gardiner, Ph.D., Department of Biology, Bryn Mawr College, and to Charles F. Branch, M.D., American College of Surgeons, Chicago, Illinois, for their help and advice in working out this method.

AN AZOCARMINE STAIN FOR DIFFERENTIAL CELL ANALYSIS OF THE RAT ANTERIOR HYPOPHYSIS

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ABSTRACT.—A method of staining is described which is especially designed to facilitate differentiation of the cell types of the rat anterior hypophysis. Fixation in Zenker-formol solution is recommended. Pre-staining of the nuclei by a short immersion in alum hematoxylin is followed by mordanting in anilin alcohol and a 45 minute period of staining in azocarmine solution at 60°C. The counterstains, acid green and orange G, are dissolved in clove oil to avoid destaining of the azocarmine.

Innumerable methods of staining the hypophysis for various cytological studies have been used by different investigators. The present technic is one which has been developed primarily for ease of differentiation of the basic cell types for quantitative cell counts.

The type of fixation of the hypophysis markedly influences the percentage of acidophiles, so it is important to consider this factor in comparing the cell counts of different investigators. Fixation in different technics can be reduced to three types of fixatives: those containing mercuric bichloride, without acetic acid (Zenker-formol); those without mercuric bichloride but with acetic acid (Bouin's); and those which contain osmic acid. Each has its own advantages and disadvantages. The Zenker-formol type of fixation gives excellent color differentiation in subsequent staining and for ease in distinguishing alpha cells (acidophiles, eosinophiles) is probably unexcelled. A criticism of this fixative when used for differential cell counts, however, is that it preserves mitochondria, which are difficult to distinguish from acidophilic granules. Bouin fixation is good, probably giving least distortion of the cells, but does not provide as sharp tinctorial differentiation in subsequent staining. A questionable advantage in counting technics is the absence of mitochondria which have been dissolved by the acetic acid. Osmic acid methods are excellent for cytological detail, especially for Golgi apparatus, but are of little use in quantitative differential counts because of variability and tissue damage.

It is interesting to speculate on the reasons for differences in alpha

cell percentages of control rats in the work of different investigators. The type of fixation seems to be fundamental in determining the approximate control value: 32-40% alpha cells (Wolfe, 1935, using Regaud's fluid; Pfeiffer, 1937; Finerty and Briseno, 1948, using the Zenker-formol type): 24-27% (Baker and Everett, 1944; Finerty, Meyer and Marvin, 1944, using Bouin fixation). Perhaps some of the difference can be attributed to difficulty in discriminating between acidophilic granules and mitochondria. However, after making cell counts of rat pituitary glands under identical conditions with only the difference in fixation, it seems unlikely that this difficulty can explain the consistently characteristic percentages obtained. This variation in response to different fixing agents may be a clue, indicating the presence of two functionally different acidophiles.

Beta cell (basophile) percentages are similar with either fixative, with possibly better differentiation of granules after Bouin fixation. Species differences certainly account for some variation in the response to staining, possibly due to slight pH differences. This has been discussed by Mellgren (1944) who allows for difference in stainability of the rat and mouse hypophysis by slight variation in pH of the solutions. The present method has been designed primarily for the rat hypophysis, since the majority of quantitative cellular counts have been made in this animal, and discussion of the methods in this paper is generally applicable to the rat.

Azocarmine staining of acidophilic granules is excellent for sharp differentiation, and is well exemplified by the Koneff modification of Heidenhain's azan method (Koneff, 1938). Prestaining of the nuclear membranes by a short immersion in alum hematoxylin brings out the nuclei sharply, making counting the total number of cells in each field much easier.

Solution of the counterstain in clove oil provides a method which allows adequate staining of the beta cell granules, without destaining the alpha cell granules. Azocarmine is rapidly destained in aqueous or alcoholic solutions (except absolute alcohol). Thus a shorter period of staining in azocarmine is allowed than in some other technics.

Each of the preferred methods which render differential cell counts easier and more accurate has been incorporated in the method which is presented in detail:

1. Fix in Zenker-formol, 6-12 hours

Potassium bichromate.....	25 g.
Mercuric bichloride.....	50 g.
Ringer's solution.....	1000 ml. (or 0.9% saline)

Add 1 ml. neutral formalin/10 ml. solution at time of using.

2. Wash 6-12 hours in running water.

3. Dehydrate in alcohols:
30, 50, 70, 80, 95%— $\frac{1}{2}$ hr. each
100%—2 changes, 1 hr. each.
4. Clear:
 - a) absolute alcohol—cedarwood oil (equal parts), 1 hr.
 - b) cedarwood oil, 1 hr. or overnight.
 - c) xylene, 15 min.
5. Infiltrate in hard paraffin (56–58° C.):
 - a) 1 change into paraffin (5 min.)
 - b) 3 changes—($\frac{1}{2}$ hr. each)
 - c) embed in hard paraffin
6. Section at 4μ .
7. Remove paraffin with xylene, 2 changes, 3 min. each.
8. Two changes absolute alcohol, 3 min. each.
9. Alcohol (95%), 3 min.
10. Distilled water, 3 min.
11. Lugol's solution (4 I: 6 KI:100 H₂O), 3 min.
12. Sodium thiosulfate (0.5% aqueous), 3 min.
13. Distilled water, 3 min.
14. Alum hematoxylin¹, 30 sec.
15. Tap water (wash)
16. Distilled water, 3 min.
17. Alcohol (80%), 3 min.
18. Anilin alcohol, 15 min.

Aniline oil	1 ml.
95% alcohol	1000 ml.
19. Stain in azocarmine at 60°C., 45 min.

Azocarmine G ²	1 g.
Distilled water	100 ml.

Warm and allow to cool to room temperature

Filter with filter paper (all day). Add

Glacial acetic acid	4 ml.
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to filtrate. (This stain may be used over again repeatedly; but should be placed in oven 1 hr. before using.)
20. Rinse in distilled water.
21. Differentiate in anilin alcohol (sol. 18) 2–3 min.
22. Wash in acid-alcohol, $\frac{1}{2}$ –1 min.

Glacial acetic acid	10 ml.
Alcohol (95%)	1000 ml.
23. 5% phosphotungstic acid, 1 hr.
24. Dehydrate in alcohols:

70% alcohol 2 min.

95% alcohol 2 min. (not longer, or azocarmine will be removed.)

Absolute alcohol 2 min. (may remain in this solution temporarily.)

¹Hematoxylin, National Aniline, Cert. No. NH3.²Azocarmine G, Grubler, 1941.

25. Counterstain in acid green solution, 5 min.

Acid green ³	0.1 g.
Orange G ⁴	0.5 g.
Clove oil	100 ml.

Use fresh stain for each batch of about 20 slides.

26. Xylene, 1 min.

27. Two changes xylene, 30 min. each

(Clove oil must be completely removed with xylene in order to prevent further decoloration)

28. Mount in Clarite.



FIG. 1

Photomicrograph of the anterior pituitary gland of a 46-day-old male rat, stained by the technic described. The cells with dark cytoplasm are acidophiles; the larger cell near the center of the field is a basophile in which the flocculent granules have been stained light green.

After the above staining, alpha cell granules are purplish red; beta cell granules are light green; nuclear membranes are sharply defined; mitochondria are orange-red; red blood cells are brilliant orange; Golgi apparatus shows as negative image in both alpha and beta cells; chromophobes show little or no cytoplasm, which is colorless to pale green. Fig. 1 is a photomicrograph of the anterior pituitary of a 46-day-old male rat stained by this method.

³Acid green L extra, National Aniline, Lot 10723.

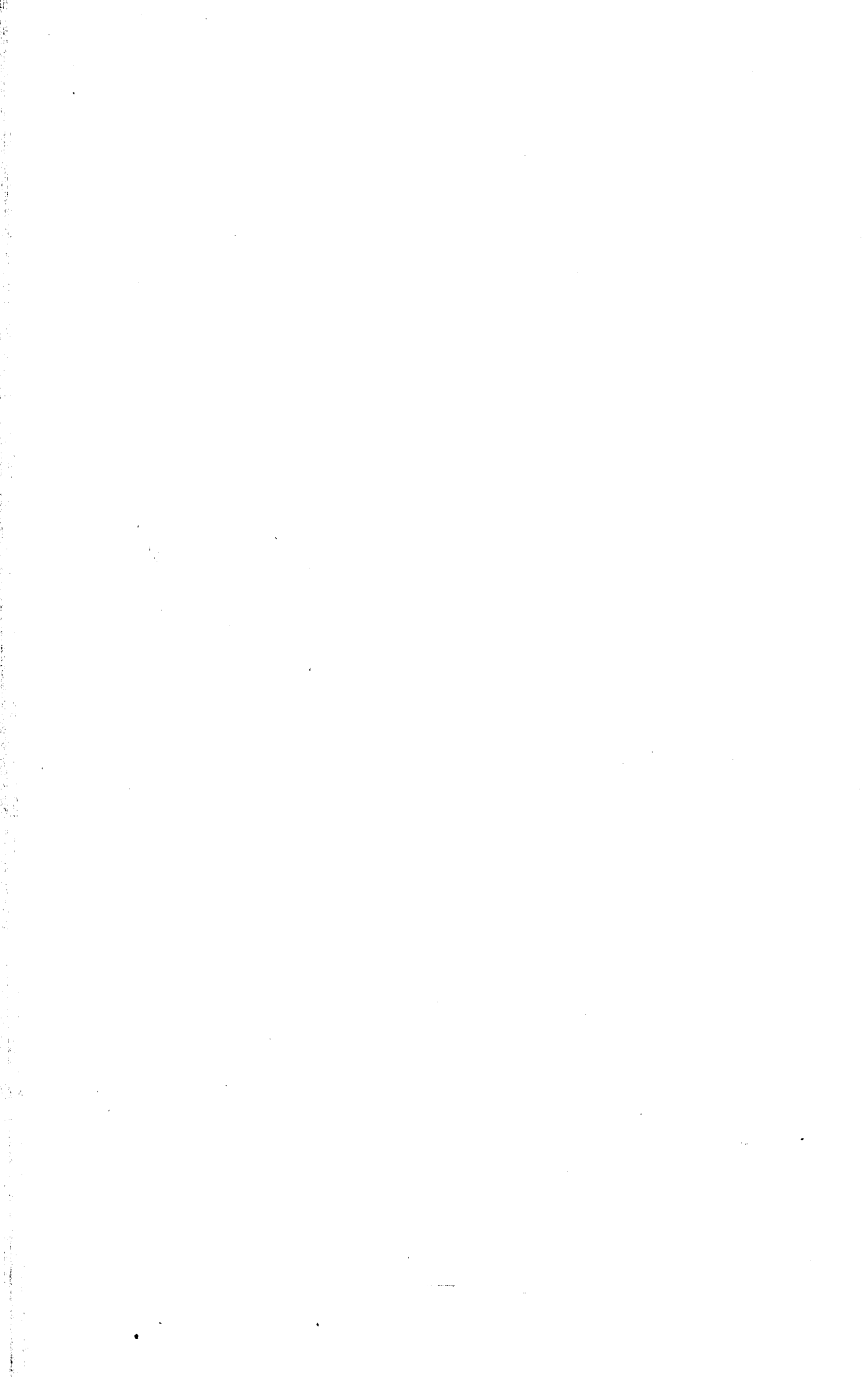
⁴Orange G, National Aniline, Cert. No. 11.

The following counting method (a modification of that of Rasmussen and Herrick, 1922) is recommended for differential cell counts using this stain. The rat pituitary gland should be sectioned in a horizontal plane. All the cells are counted in every fifth field in each of three horizontal sections at equidistant levels in the gland. Consistent enough results can be obtained among the three sections so that it is possible to get quite accurate counts using only one section through the center of the gland. Most variation in cell percentage exists between the central and peripheral regions, which are both sampled adequately by a center section. With this method, about 1200 to 1400 cells can be counted, using one central section of the pituitary gland of a 41-day-old rat (pituitary weight about 5.0 mg.)

Good results have been obtained using this stain on other glandular tissues, such as the thyroid and adrenal glands and the pancreas.

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THE HISTOCHEMICAL DETECTION OF IRON IN TISSUES¹

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ABSTRACT.—The fixation and staining of iron in tissues is discussed. Procedures for demonstrating iron in hemoglobin and nuclei are also briefly considered.

Lillie's formalin buffered at neutrality gave the optimal fixation for iron. The Prussian blue method was preferred to the Turnbull blue. Lison's procedure of the former, slightly modified, gave the most satisfactory results. When a procedure is required that employs non-iron-containing reagents, Macallum's or Mallory's hematoxylin and Quincke's ammonium sulfide method are useful. The former, though not entirely specific, is preferable under controlled conditions when the quantities of iron are small. Hemoglobin iron in paraffin sections can be demonstrated by the usual procedures for iron after previous exposure of the section to peroxide, as recommended by Brown. The property of nuclei to adsorb iron from inorganic sources and from hemoglobin can readily be shown; caution is required in interpreting the iron detected in nuclei after Macallum's sulfuric acid hydrolysis.

In medical literature there have been comparatively few discussions of the methods for the detection of iron in histological sections. Lison (1936) presents the histochemical aspects of various procedures, while Gomori (1936) has written a critique of the Prussian blue (Perls), the Turnbull blue (Tirman and Schmeltzer) and the iron sulfide (Quincke) methods. Lillie (1948) includes a consideration of various fixatives and stains for iron. The present report is an effort to evaluate the more important of these methods and others that have proved useful.

Tissues containing relatively large amounts of iron as test objects were represented by spleen and liver of mouse and rat, cat's placenta,

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smears and clots of rat's blood, bone marrow of monkeys infected with *Plasmodium knowlesi*, human apocrine sweat glands, foci of old hemorrhage and interstitial deposits of iron in the walls of human cerebral arteries and choroid plexus, and organs from a patient with hemochromatosis. Numerous tissues not containing conspicuous amounts of iron were used as controls.

Fixatives. The present investigation has confirmed the findings of Lillie (1948) that 10% formalin buffered at neutrality is superior to other fixatives for the detection of iron by chemical methods. By its use, more iron is preserved with less diffusion than with unbuffered formalin, alcohol-formalin, Zenker's or Helley's fluid. Tissues fixed in Zenker's and Helley's fluids, of course, must not be washed in tap water since this introduces a source of contamination with soluble iron that can be adsorbed by the nuclei (see below). Alcohol-ammonium sulfide as recommended by Hall (1896) preserves hemosiderin well but hemolyzes the red blood cells.

Detection of Reactible Iron. The chemical methods for the detection of iron depend upon the formation of either specific compounds or colored lakes. The Turnbull blue and Prussian blue reactions belong to the former group, whereas Macallum's (1898) and Mallory's (1938) hematoxylin methods as well as Humphrey's (1935) dinitroresorcinol procedure are examples of the latter. It will be recalled that in the Turnbull blue procedure of Tirmann and Schmeltzer (Lison, 1936) the section is exposed to ammonium sulfide which converts the ionizable iron to ferrous sulfide before it is brought into contact with ferricyanide to form ferro-ferricyanide. Ferrous iron, such as may occur alone or in combination with ferric iron in the interstitial deposits mentioned above in formalin-fixed specimens, is detectable by omitting the ammonium sulfide. The Prussian blue procedure depends upon the reaction of ferric iron as in hemosiderin and most other sites with the ferrocyanide radicle to form ferri-ferrocyanide. The sensitivity of certain of these reactions is very high; for example, Lison (1936) states that the Prussian blue procedure as used in microchemistry is capable of detecting 0.002 γ of iron.

There are certain differences in the histological results with these two procedures. Gomori (1936) pointed out that the iron particles as demonstrated by the Turnbull blue precipitate are larger than when revealed as iron sulfide by Quincke's reaction (cf. Lison, 1936); such distortion of the actual particle size is not produced by the Prussian blue method. The present studies have confirmed Gomori's observations. It has further been found that frequently less iron is revealed by the Turnbull blue than by the Prussian blue method. It

is felt, therefore, that the latter is the better procedure. In carrying out the Prussian blue reaction, the simultaneous rather than sequential use of HCl and ferrocyanide is better since under this condition the iron is precipitated by the ferrocyanide as soon as it is dissolved from the original deposit by the acid; only thus can a maximal reaction be obtained.

Different concentrations of the reagents and conditions for carrying out the Prussian blue procedure have been specified by each investigator: Gomori (1936), equal parts of 20% HCl and 10% sodium ferrocyanide; Lison (1936), equal parts of 2% HCl and 2% sodium ferrocyanide; Lillie (1948), equal parts of 5% acetic acid and 2% sodium ferrocyanide heated to 80°C. In the present study, different combinations of 2-20% HCl with equal parts of 2-10% sodium ferrocyanide were tested on hemosiderin-type iron as well as on that which occurs pathologically in interstitial deposits in the human choroid plexus and cerebral arteries. The concentrations recommended by Lison (1936) have been found to be superior to the others. The best results have been obtained when the exposure time was increased from the 15-30 minutes recommended by Lison, to 1 hour, replacing with freshly prepared solution at the end of the first half hour to obviate any break-up of the ferrocyanide radicle. Extending the time beyond 1 hour did not increase the intensity of the deposit. More iron has been revealed by this modification of Lison's method than by 5% acetic acid with 2% ferrocyanide. It demonstrates hemosiderin-type iron as well as Gomori's method and is capable of revealing deposits of interstitial iron at times unstainable by the more concentrated reagents of the latter method.

The solutions of both the Prussian blue and Turnbull blue reactions are unstable, turning pale green after an hour or so as the iron, split off from the ferro- or ferri-cyanide radicle and changed in valence, reacts with intact radicles. It is observed that strongly acidic objects in sections left in such solutions for several hours are selectively stained blue: e.g. mast cell granules, nuclei, and the stratum corneum of the rat. The iron is undoubtedly first removed from the unstable combination in the radicle by the acidic groups of the objects and then promptly reacts with intact radicles, forming a blue precipitate at these loci. This never occurs, however, if the solution is renewed every half hour during such a prolonged exposure. The Prussian blue and Turnbull blue reagents, therefore, should always be made immediately before use, and the section exposed no longer than the time prescribed for the particular method.

A staining reagent which does not itself contain iron is sometimes

needed. Ammonium sulfide, introduced by Quinke in 1868 (cf. Lison, 1936), meets this requirement. Its histochemical specificity under ordinary conditions where other heavy metals are not present is equal to that of the Turnbull and Prussian blue reactions. The method is satisfactory when the iron is abundant (giving a grayish-black, green, or brown iron sulfide) but it is not as good as the ferro- and ferricyanide procedures for the detection of very small amounts since the resulting color under these conditions is yellowish-brown and difficult to recognize, even in comparison with unstained control sections.

Macallum's or Mallory's hematoxylin methods can also be used for this purpose, an insoluble blue-black or gray iron lake being formed. Although this reaction is not specific for iron—because salts of calcium interfere (Lison, 1936)—it is very sensitive, and is useful under controlled conditions. Hemosiderin iron may stain only brown in tissues fixed in either alcohol or formalin. If the section is exposed first to ammonium sulfide, however, hemosiderin iron is stained blue-black or grey. After such pretreatment it is clearly seen that the pale brown objects (iron sulfide) are the ones which react with the hematoxylin. Thus the specificity of the procedure in the particular specimen can be controlled.

Humphrey's (1935) dinitrosoresorcinol procedure which also depends upon the formation of a lake with iron is less sensitive than Macallum's hematoxylin. The brown color imparted to the background by this dye acts as a counterstain for the blue-green iron lake, but also obscures some of the smaller iron deposits.

The use of the synthetic resin, Clarite, as a mounting medium obviates the fading of the Prussian and Turnbull blue preparations, so common with balsam.

If a counterstain is desired with the Turnbull blue, Prussian blue, or Macallum's hematoxylin methods, Mayer's paracarmine (Mallory, 1938) is satisfactory; it is not alkaline and does not adversely affect the ferro-ferricyanide or ferri-ferrocyanide deposits. If iron is present in traces only, a counterstain is to be avoided as it can obscure small deposits. With the Turnbull blue and Prussian blue reactions a pink filter interposed between the light source and the microscope aids materially in the detection of small amounts of the blue precipitate; this is especially useful with sections which have not been counterstained.

Detection of iron in hemoglobin and in nuclei. Lillie (1948) stated that the iron in hemoglobin altered by acid may sometimes be stainable by the usual procedures for iron. Brown (1911) described the

use of hydrogen peroxide on formalin-fixed unembedded material for this purpose; this has been found satisfactory for paraffin sections as well. Brown recommended his method in addition as a means of staining the iron of hematin (as in malaria); this has not proven successful on embedded material.

Macallum (1895) (or cf. Lison, 1936) demonstrated that hydrolysis of sections in sulfuric acid resulted in the nuclei subsequently being stainable with the usual reagents for the detection of iron. There seems to be little doubt that it is iron which is thus revealed by the ferro- or ferricyanide solution rather than a false reaction, since similar results are obtainable when Quincke's ammonium sulfide method is used to detect the iron. A controversy exists, however, as to whether the iron so stained is of intrinsic nuclear origin or whether it has been adsorbed by the nuclei from other sources. The investigators with wide experience in micro-incineration do not agree in all respects concerning the presence of iron in nuclei (cf. Scott, 1933; Horning, 1945; Policard, 1941-42; MacCardle, et al., 1943). Wiener (1916) has shown that nuclei have an affinity for iron from various sources: the reagents, glassware, hemoglobin, and the microtome knife. While this particular problem was not an objective of the present study, certain relevant observations have been made. After Macallum's sulfuric acid hydrolysis, iron has been demonstrated in various kinds of nuclei with the Prussian blue and Quincke's ammonium sulfide methods. No more than the usual precautions (use of distilled water, glass instruments except for the microtome knife), however, were taken for the avoidance of iron. The adsorption of iron by nuclei has been readily demonstrated in several ways. The nuclei of cells in the vicinity of large amounts of hemosiderin exhibit iron at times by the Prussian blue or hematoxylin methods without even requiring previous sulfuric acid hydrolysis. If the section be hydrolyzed, all of the nuclei then become stained, but those nearer the hemosiderin are darker blue than the remainder. It is not known whether this adsorption occurs during life or while the tissue is in the fixative. Inorganic iron from tap water or as ferric chloride can be adsorbed from solution by fresh or fixed tissues and is stainable in greater quantities in the nuclei than in the cytoplasm by the Prussian blue method without previous hydrolysis. Hemoglobin iron from laked red blood cells is also adsorbable by nuclei in either fresh or previously fixed tissues, but requires Macallum's hydrolysis to reveal it. These findings demonstrate the need for caution in interpreting the iron shown in nuclei by this procedure. It should be noted that sulfuric acid hydrolysis reduces or removes hemosiderin and hematin (as in malaria

marrow) as well as iron occurring in the granules in the epithelium of apocrine sweat glands. Hemoglobin iron is not stainable by the Prussian blue method after hydrolysis.

METHODS FOR DETECTION OF IRON FOUND MOST SATISFACTORY IN THE PRESENT STUDIES

Material is fixed in 10% formalin buffered at neutrality (4 g. monohydrated acid sodium phosphate and 6.5 g. anhydrous disodium phosphate per liter) for 24 hours, as recommended by Lillie.

Prussian blue method: Equal parts of aqueous 2% sodium ferrocyanide and 2% HCl are mixed immediately before use, and filtered. Sections are exposed to this fluid for a total of 1 hour at room temperature (instead of 15–30 minutes given by Lison); it is replaced with freshly prepared solution at the end of the first half hour.

Hematoxylin method: Hemosiderin iron, whether fixed in formalin or alcohol, may be stained an unsatisfactory brown, while the interstitial iron is blue-black with hematoxylin. It has been found, however, that if the section is exposed to 10% aqueous ammonium sulfide for 1 hour at room temperature, before staining, the hemosiderin also forms a blue-black hematoxylin lake. The hematoxylin method as used contains details of both Macallum's and Mallory's methods. The solution should be prepared immediately before use, as follows: dissolve 5–10 mg. of ether-washed hematoxylin crystals in a few ml. of absolute alcohol; add this to about twice the volume of boiled, distilled water. Sections are stained 1–2 hours. The excess hematoxylin is removed by washing in equal parts of absolute alcohol and ether for 30 minutes, and then rinsing in distilled water.

Counterstain: If desired, after either of the above stains, one may counterstain with Mayer's paracarinine (Mallory); but, when only traces of iron are being sought, the counterstain should be omitted.

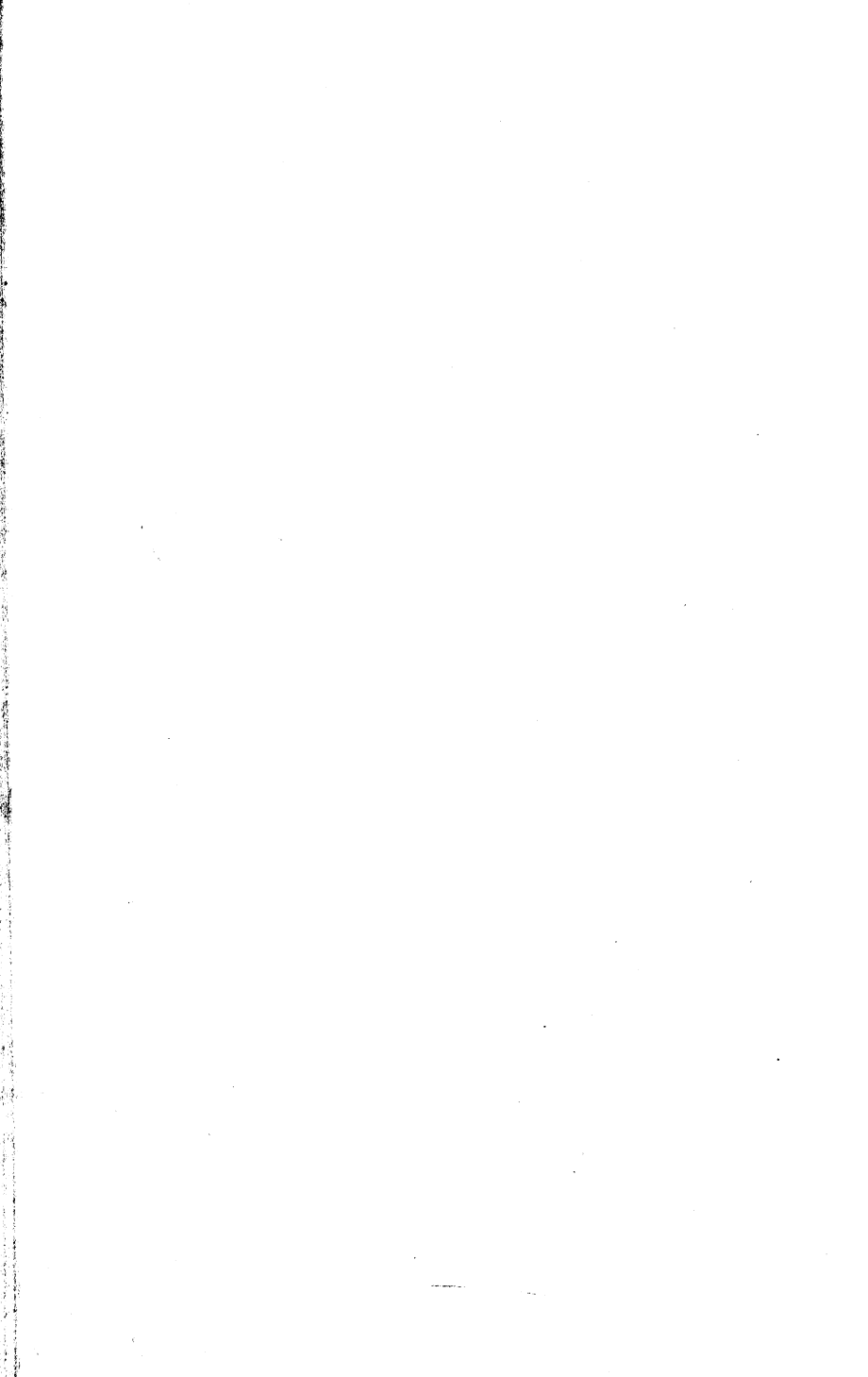
Mounting: Clarite should be used, to obviate the fading of ferri-ferrocyanide encountered after using balsam.

A pink filter on the light source aids materially in detecting small amounts of ferri-ferrocyanide and is especially useful when the section has not been counterstained.

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GOLGI'S DICHROMATE-SILVER METHOD

1. EFFECTS OF EMBEDDING

2. EXPERIMENTS WITH MODIFICATIONS

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ABSTRACT.—Paraffin embedding was found to be satisfactory for brain stained by a modification of the Golgi dichromate-silver method. Nitrocellulose embedding caused fading in a few specimens. Several modifications in which the tissue was impregnated with silver nitrate before treating it with potassium dichromate were investigated. The following one is recommended. Fix pieces of brain 5–6 mm. thick for 2 days in: silver nitrate, 0.5%, 90 ml.; formalin, comml. unneutralized (37–40% gas), 10 ml.; pyridine, pure, 0.05–0.1 ml. Mix in the order given and test for pH with brom cresol purple. A pH of 5.5–6.0 is about optimum and the amount of pyridine added can be varied to adjust it. A slight turbidity of the fixing fluid may be disregarded, but precipitation indicates too much alkalinity. Rinse the tissues with distilled water and place them in a mixture of potassium dichromate, 2.5%, 100 ml. and osmic acid, 1%, 1 ml., for 3–5 days. Wash in water, dehydrate with alcohol and embed in soft paraffin for thick sectioning. Greater intensity of staining (but with an increase in precipitate) can be secured by rinsing the blocks after the dichromate treatment and resilvering in a 0.5% solution of silver nitrate for a day or two, then washing, dehydrating and embedding. This modification of the Golgi method was worked out on brain of adult rat, guinea pig, cat and monkey. Results with fetal material were not good. All solutions used were aqueous, and staining was done at room temperature.

1. EFFECTS OF EMBEDDING

Many consider embedding inimical to the success of the Golgi (1875, 1894) silver-dichromate method for nervous tissue. Among these, Lee (1937) suggests celloidin for friable tissues, but advises against prolonged dehydration time, use of xylene for clearing, and paraffin embedding.

Strong (1896) found that the resistance of the stain to alcohol in-

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In testing several variations of the rapid Golgi method, our best success occurred with a modification of Fish's technic (1895) which consisted of fixing 3 days in $K_2Cr_2O_7$, 2.5 g.; formalin (conc.), 1 ml.; 1% osmic acid, 1.0 ml.; distilled water, 100 ml., and silvering in 0.5% $AgNO_3$ (aqueous) for 2 days. Attempts to minimize precipitate in the tissue by coating the blocks with agar jelly and by washing at various stages of the process were unsuccessful. Experiments with refrigeration during fixation and staining likewise gave no hope for improvement.

FORMALIN-SILVER-DICHROMATE SEQUENCE

Fresh brain placed in dilute $AgNO_3$ solution for 24 or 48 hours, without a preliminary fixation in formalin, and then treated with dichromate, did not give any worthwhile staining. Hill (1896) had similar failures on silvering before chromation. Fixation in 10% unneutralized commercial formalin for time intervals of 1 hour to 10 days indicated that no appreciable effect on staining was obtained when the fixation time was 4 hours or less. Progressive inhibition of staining occurred from about 24 hours onward, so that almost no silvering occurred in tissues fixed for 72 hours or more. The optimum time for fixing halves of rat or guinea pig brains was judged to be between 12 and 18 hours. Several modifications of Bouin's fluid and also Cajal's formalin ammonium bromide solution were tried, but no impregnation of the Golgi type was obtained after them.

Silvering, following formalin fixation without washing, was tried with 0.5, 0.75 and 1.0% aqueous solution of $AgNO_3$. This step appeared to be uncritical provided sufficient time were allowed for penetration of the block. A concentration of 0.5% acting for 36 hours on small blocks, 3-5 mm. thick, to 48-72 hours for blocks 6-8 mm. thick gave optimum silvering. Silver chlorate and silver sulfate were tried, but although the chlorate gave an impregnation similar to the nitrate, no advantage in its use was apparent. The sulfate was somewhat less active than the nitrate. Hill (1896) tried the acetate and nitrite but found no advantage in their use.

A 2.5% solution of $K_2Cr_2O_7$ was used after the silvering, and the blocks of tissue left in it for 48 to 72 hours. The blocks were then washed, dehydrated and embedded in paraffin, and sections 50 μ thick cut therefrom for histologic study.

COMBINED FORMALIN- $AgNO_3$ FIXATION FOLLOWED BY $K_2Cr_2O_7$

When a mixture of 0.5 g. of $AgNO_3$ in 100 ml. of 10% formalin was used for fixation, the inhibition of staining caused by fixation for more than 24 hours in formalin alone was not evident. A fixation

time at room temperature of 48–72 hours was optimum. The addition of alcohol to make 10%, 20% and 30% in the final concentration of the fixing fluid was tried without significant benefit. The impregnation of nerve fibers was enhanced to some extent, but the amount of spurious precipitate in the final stain was increased. The addition of pyridine resulted in instability of the mixture when the amount added was enough to increase the pH beyond 6.0. Acidification of the fixing fluid to a pH of less than 5.0 by addition of acetic acid resulted in failures, hence checking (colorimetrically) the pH and adjusting it to 5.5–6.0 is recommended.

After fixation, blocks were rinsed with distilled water and transferred to either a 2.5% solution of $K_2Cr_2O_7$ or to a like solution to which sufficient osmic acid had been added to give 0.01% in the final concentration. Osmic acid seems to exert a favorable catalytic effect on the staining reaction.

Experiments in which the tissues were left in the dichromate or dichromate-osmic solution from 1 to 10 days were made. Little change occurred after 3 days. When fixing and silvering are done together, most of the reaction with the dichromate solution occurs within the first 2 days after transferring the tissues from the fixing fluid into it.

EFFECTS OF VARIOUS TREATMENTS AND REAGENTS

1. *Washing.* Soaking blocks in distilled water between silver and dichromate for more than 0.5 hour caused progressive lightening of the stain as well as a reduction in the amount of precipitate.

2. *A second silvering.* Return of blocks to $AgNO_3$ solution after dichromate resulted in an increase of staining and depth of penetration for neural elements and blood vessels, and formed more precipitate as well. Washing between dichromate and silver made the precipitate worse. A second silvering given after tissues had remained in dichromate for intervals of 1.5, 3.5, 4.5 and 10 days showed no certain effect after the 2.5-day period. The 1.5-day specimen showed less staining from the resilvering, but the interval in dichromate was not critical.

3. *Oxidizing and reducing agents.* The addition of 2% $KClO_3$ to the formalin, when used for fixing prior to silvering, inhibited all staining. An addition of either hydroquinone, tannic acid or gallic acid in 0.1% concentration to the formalin resulted in an intense blackening of the tissue, but neural elements were masked by precipitate. When they were added in only 0.005% concentration, staining was enhanced but any advantage was outweighed by excessive precipitate.

4. *Effects of heat.* All three stages of the procedure (fixation, silvering and dichromate treatment) were tried individually at 5°, 25° and 37°C. No advantages were seen in the refrigerator and incubator treatment over staining done at room temperature. Stains were darker when made at 37° but the character and quality was similar to that made at lower temperatures.

5. *Surface tension.* The addition of 0.001% sodium lauryl sulfonate (purified "Dreft") seemed to give an increase in the depth of penetration of the stain into the tissue block and also enhance staining of nerve cells. The amount of precipitate increased also, hence any net improvement was questionable.

DISCUSSION OF RESULTS

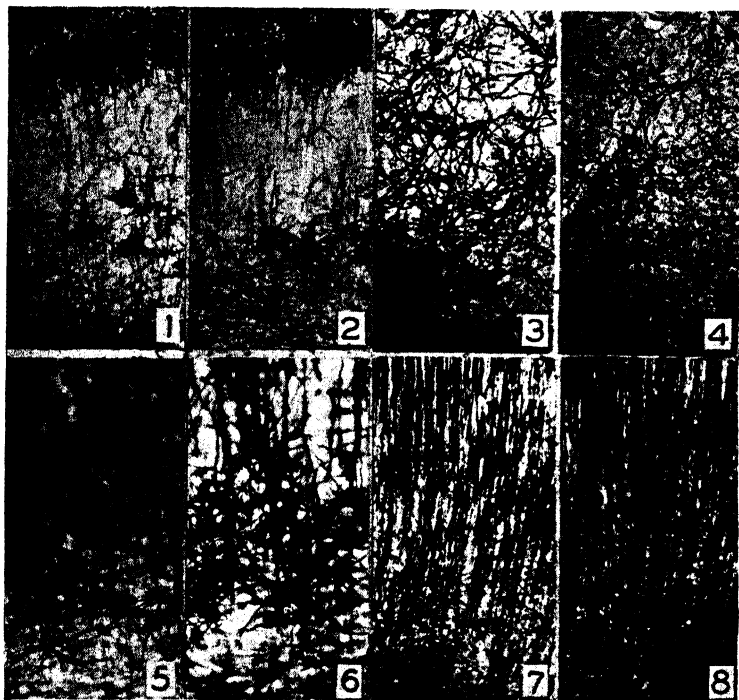
The general appearance of the impregnation is not altered by reversing the order of applying the reagents. Neural elements stain a deep brown to black against an amber background. The amount of precipitate formed inside the tissues varies from block to block, and from much at the surface to little in the interior. Staining of capillaries is often annoying, but it was not eliminated in the one cat brain that was perfused.

When brains were fixed in formalin before silvering, more pericellular shrinkage, with subsequent accumulation of precipitate (Fig. 1 and 2), was seen than that which occurred when fixing and silvering were done simultaneously. Diffuse, finely granular precipitate tended to increase with increase in the time allowed for silvering. Attempt to minimize precipitates by washing the blocks during processing and by varying the concentration of the dichromate solution (0.5%, 2.5% and 15%) were of no avail.

Staining in the cerebellum was limited to granule cells with their dendrites, an occasional Purkinje cell complete with dendrites and axon stub (Fig. 10 and 12), nerve fibers, and a few Golgi type II cells. Spinal cord, in the few specimens tried, did not give satisfactory staining, although an occasional anterior horn ganglion cell stained in one spinal cord from an adult cat. The best and most consistent staining was seen in the cerebral cortex. Pyramidal cells in the cortical layers III and V (Fig. 13), their dendrites, and often axons as well, stained in nearly all specimens. Subpial dendritic ramifications (Fig. 11) were brought out conspicuously in some areas of many specimens. Astroglia (Fig. 14) were stained rather frequently, especially in white matter.

It is recognized that every modification of the Golgi method may

have a special application wherein it excels other modifications. The method suggested below is given as one that has produced the largest number of Golgi-type stains when applied without an objective of staining a particular structure. Securing a favorable staining zone in



Explanation of figures 1-8. Section thickness: approximately 50 μ . Magnifications: 1, 2, 7 and 8; $\times 50$; 3, 4, 5 and 6; $\times 100$. The odd numbers are of sections from blocks of unembedded Golgi-stained rat cerebral hemispheres. The even numbers are of sections from paraffin embedded parts of the same blocks, either of the opposite hemisphere (1 to 4) or of one half of the same hemisphere (5 to 8). Each pair of figures (1, 2; 3, 4; etc.) represents material from a different rat. Figures 1 and 2 show dense overstaining of the subpial area (top of figures) and excessive precipitation about the bodies of pyramidal cells. Paraffin embedding might be blamed for such results if the unembedded control did not show the same features. Figures 3 and 4 were taken from an anatomically unidentified subcortical cell mass, 5 and 6 show groups of pyramidal cells, and 7 and 8 show an area of pyramidal cell dendrites which contain an abundance of fine processes that do not seem to have been damaged by paraffin embedding.

a block of tissue depends frequently on the depth of the site within the block. Paring away undesired portions of the fresh tissue to within 1-2 mm. of the area one hopes to stain is a means of facilitating such staining.



Explanation of figures 9-16. Section thickness: approximately $50\ \mu$. Photomicrographs of sections of this thickness fail to show all of the details of stained structures, for example, axons on many nerve cells were seen with higher magnification and focusing but rarely appeared in the focal plane of the photograph.

9. Cerebral cortex, rat; large ganglion cells and dendrites. $\times 200$. AgNO_3 , 6 hr.; formalin- AgNO_3 , 4 hr.; $\text{K}_2\text{Cr}_2\text{O}_7$ - OsO_4 , 48 hr.
10. Cerebellum, cat; granule cells and dendrites. $\times 250$. AgNO_3 -formalin-Py, 36 hr.; $\text{K}_2\text{Cr}_2\text{O}_7$, 48 hr.
11. Cerebral cortex, rat; ganglion cells and subpial dendritic ramifications. $\times 90$. Formalin, 12 hr.; AgNO_3 , 24 hr.; $\text{K}_2\text{C}_2\text{O}_7$ - OsO_4 , 48 hr.
12. Cerebellum, guinea pig; Purkinje cells. Several axons of these cells were present but not in the plane of focus. $\times 100$. AgNO_3 -formalin-Py, 36 hr.; $\text{K}_2\text{Cr}_2\text{O}_7$, 48 hr.
13. Cerebral cortex, rat; pyramidal cells. $\times 50$. Formalin, 6 hr.; AgNO_3 , 24 hr.; $\text{K}_2\text{Cr}_2\text{O}_7$ - OsO_4 , 48 hr.

METHOD: FORMALIN-SILVER GOLGI MODIFICATION

1. Fix 48 hr. at about 25°C. in the following mixture:

Silver nitrate, 0.5 aqueous.....	90 ml.
Formalin (Conc. comml., unneutralized) ..	10 ml.
Pyridine, pure.....	0.05 to 0.1 ml.

To be mixed in the order given. If a slight turbidity occurs, it may be disregarded. A colorimetric pH test with brom cresol purple should show 5.5–6.0. If excessive precipitation occurs, the formalin may not require the addition of pyridine, hence it can be omitted.

If specimens are to be fixed by perfusion, flush out the blood with plain 10% formalin and follow with the mixture. Cut into slices of other suitable blocks to limit the thickness to 0.5 to 1.0 cm.; leave immersed in the fixing fluid about 48 hr.

2. Rinse the blocks with distilled water and place in 2.5% potassium dichromate (aqueous) to which 1 ml. of 1% osmic acid solution is added for each 100 ml. for 3 to 5 days. (The osmic acid is optional and can be omitted.)

3. Wash with water, then 50% alcohol, 1 hr. each, to remove the dichromate solution. Dehydrate with 95% alcohol and absolute, remove alcohol with xylene and embed in paraffin in the usual manner. (Soft paraffin is recommended if sections 50 to 100 μ thick are desired.)

Variations: Blocks may be allowed to remain in the dichromate solution (Step 2) for 1 to 2 weeks without harm and, more likely, they will be improved. Sections may be cut free-hand with a razor after step 2, dehydrated, cleared, and examined microscopically. If the impregnation is too weak, the blocks can be placed in a 0.5% AgNO_3 solution for 24 hours and examined again. The extent of staining is increased by this supplementary silvering, but so also is the amount of precipitate.

ACKNOWLEDGMENT

The major portion of the cost of this research was defrayed through a grant received from the Biological Stain Commission.

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14. Cerebrum, rat; astroglia and small blood vessels. $\times 90$. From the same block as No. 11.
 15. Cerebral cortex, rat; pyramidal cells and dendrites. $\times 90$. Same staining procedure as No. 11.
 16. Cerebrum, rat; hippocampal region in which the small cells of the dentate fascia appear in the middle and the ganglion cells of the stratum griseum circumvolutum are at the top and bottom of the figure. $\times 90$.

Formalin- AgNO_3 -“Dreft”-Py, 36 hr.; $\text{K}_2\text{Cr}_2\text{O}_7$ - OsO_4 , 48 hr.

Note on the schedules of technics listed above: (1) Concentrations of reagents: formalin, 10%; AgNO_3 , 0.5%; Py, 0.1%; $\text{K}_2\text{Cr}_2\text{O}_7$, 2.5%; OsO_4 , 0.01%; “Dreft,” 0.001%; all aqueous. (2) The listing is intended to show the flexibility of procedures used rather than to give any recommendation of the specificity of a given technic for the structures figured.

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THE FEULGEN-PICRIC-ACID BLOCK STAIN ADDITIONAL DATA

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ABSTRACT.—Additional experiments on staining of tissues in the block by means of picric acid and the Feulgen reaction (Lhotka and Davenport, 1947) show that a variety of tissues from both plant and animal sources will respond favorably. An attempt to combine fixation and staining into a single step was not successful for mammalian tissues, but gave fair results on frog. Fixation in sublimate-acetic was unsatisfactory for block staining, and conversely, fixation in sulfosalicylic-picric gave poor Feulgen stains on the slide. Soxhlet extraction of tissue with absolute alcohol removed Feulgen positive, non-nuclear material with the exception of that in vascular endothelium, cartilaginous matrix and elastic tissue. After such extraction, similar results were obtained with both block and slide methods of staining.

This paper supplements the preceding one (Lhotka and Davenport, 1947) by giving the results obtained from several additional variations of the technic. The variations included the use of tissues from widely different sources, alterations of the acidity of the fixing fluid, an attempt to combine fixation and staining, and the thorough extraction of tissue with lipid solvents.

MATERIALS

A variety of organs from laboratory animals, surgical specimens of human tumors, induced (methylcholanthrene) fibrosarcomas from rats, organs from earthworm, oyster and frog, and root-tips and leaves of onion were used. The fixing, staining, and other features of processing will be described individually.

FIXING FLUIDS

In two-color block staining, wherein the Feulgen reaction provides the nuclear stain, the proper degree of acid hydrolysis during fixation

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¹Publication No. 499. This paper and the preceding one (1947) comprise work done by John F. Lhotka in the course of fulfilling the requirements for the Master of Science degree.

is essential. Feulgen-Brauns (1924), in her study of conditions which affected staining on the slide, gave particular attention to the effect of hydrolysis on subsequent staining. The studies included both fixed and unfixed tissue of plant and animal origin, and the findings indicate that fixed tissues require more hydrolysis than unfixed. Also, the kind of fixative used influences the amount of hydrolysis required for the optimal "nuclear reaction". The optimum time for hydrolysis in *N* HCl at 60°C was found to be 4 minutes, but at 20°C. 24 hours were required. In *N*/10 HCl, 5 hours were required at 40°, but no tests were made at 20°.

Bauer (1932) showed that the method of fixation had a marked influence on the duration of the subsequent hydrolysis necessary for an optimum Feulgen reaction. He demonstrated also that, contrary to opinions of previous investigators, fixing fluids which contained formalin such as Bouin-Allen and Helly's could be used, and that a longer time for hydrolysis was necessary after fixation in fluids which contained dichromate or chromic acid.

Previously, we had determined empirically that fixation of fresh tissue for 24-48 hours at room temperature in an equal-parts aqueous mixture of 5% sulfosalicylic acid and saturated picric acid made nuclei Feulgen-positive. In the present experiments, the composition of the fixing fluid was varied by using mixtures of different acidity and different alcohol content. Mercuric chloride and potassium dichromate were added to some of the mixtures to see whether the character of the stain would be changed. Table 1 gives the composition of the fixing solutions wherein alcohol was used and Table 2 those which contained mineral and other acids and salts.

TABLE 1. FIXING FLUIDS WITH VARYING ALCOHOL CONTENT

No.	Picric acid	Sulfosalicylic acid	Absolute alcohol	Water	pH
	g.	g.	ml.	ml.	
1	0.75	2.5	100	0	1.10
2	0.75	2.5	95	5	1.05
3	0.75	2.5	70	30	1.05
4	0.75	2.5	30	70	1.00
5	0.75	2.5	0	100	0.90

The best Feulgen reactions occurred after fixation in the solutions listed in Table 1. Contrary to expectations, the pH of the alcoholic solutions differed very little from that of the aqueous. The contrast between the nuclei and background stain was somewhat greater after fixation in solutions which contained 70% alcohol or more. The time required for fixation was found to have an optimum of about 36

hours in all concentrations of alcohol and this is in harmony with effect on hydrolysis indicated by the similarities of pH.

The staining after fixing in solutions listed in Table 2 was, with the exception of fluid No. 3, inferior to that which followed fixation in picro-sulfosalicylic mixtures. A fair Feulgen reaction was obtained after 12 hours fixation in picro-sulfuric acid, since the pH of this fluid was about 0.5, but regardless of the time of fixation, the histological picture was poor. The formic acid mixture gave a weak Feulgen reaction and poor tissue preservation. The addition of either dichromate or bichloride tended to weaken the Feulgen reaction.

TABLE 2. FIXING FLUIDS WITH VARIATIONS IN ACID AND SALT CONTENT

No.	Kind and amount of acid added	HgCl ₂	K ₂ Cr ₂ O ₇	pH
	final conc.	g. per 100 ml.		
1	1% formic	—	—	1.65
2	5% formic	—	—	1.45
3	2.5% trichloroacetic	—	—	1.10
4	0.66 N H ₂ SO ₄	—	—	0.50
5	0.66 N H ₂ SO ₄	1	—	0.50
6	2.5% sulfosalicylic acid	5	—	0.85
7	2.5% sulfosalicylic acid	—	2	0.90

Picric acid to saturation was added to all of these solutions.

COMPARISON OF THE BLOCK AND SLIDE REACTIONS

Three series of specimens of intestine and spleen were fixed as follows:

1. Feulgen's fixing solution (saturated HgCl₂ in 5% acetic acid).
2. 10% formalin
3. Picro-sulfosalicylic solution (No. 5, Table 1).

TABLE 3. FEULGEN'S REACTION AFTER HYDROLYSIS EITHER DURING FIXATION, AFTER MOUNTING OF SECTIONS, OR BOTH.

Fixation 48 hr. in:	Staining technic	Hydrolysis effected by:			Character of nuclear reaction
		The fixing fluid	24 hr. in P-SS a.f.*	5 min. 60°C. N HCL	
1. Picro-sulfosalicylic acid	block	yes	no	no	strong medium weak
	slide	yes	no	yes	
	slide	yes	no	no	
2. 10% Formalin	block	no	yes	no	{atypical, all tissue components stained typical, strong
	block	no	no	no	
	slide	no	no	yes	
3. Sublimate acetic	block	no	yes	no	weak weak strong
	block	no	no	no	
	slide	no	no	yes	

*P-SS a.f. = picrosulfosalicylic acid after fixation.

The subsequent treatments and results of staining with Schiff's (Feulgen's) reagent are shown in Table 3. The tissues fixed in formalin were washed thoroughly for several days in running water to remove all formalin not bound chemically.

The findings listed show that staining in the block will not follow either fixation in formalin or sublimate acetic even though the tissue is subjected to a second fixation, or hydrolysis, in P-SS. Conversely, tissue fixed originally in P-SS failed to respond well to staining on the slide either with or without additional hydrolysis. This suggests that the transition of the nucleoprotein involved from the natural to the denatured state exerts a strong influence on the subsequent restoration of the color of the leucofuchsin, and that the process of hydrolysis on the slide is not directly comparable to the action of the acid during fixation.

ONE SOLUTION BLOCK STAIN

Sulfosalicylic acid, 5 g. per 100 ml., was substituted for the 10 ml. in *N* HCl in the Feulgen reagent. Just before use as a fixing and staining solution, an equal volume of picric acid (saturated aqueous solution) was added. Thus, the final concentrations of the various reagents were the same as they were in the two-solution fixing and staining procedure except for the omission of the HCl. Tissues were fixed and stained in the single solution for 24-48 hours.

Unfortunately, the procedure is better in theory than in practice. Fairly well differentiated nuclei and cytoplasm were obtained with plant tissue and with tissues from frog, oyster, and earthworm. Mammalian tissues gave weak Feulgen reaction with nuclei partially stained by picric acid. It seems that nucleoprotein must be denatured before staining for best results, but the fact that even a mediocre stain of the Feulgen type occurred shows that the reaction can occur concomitantly with fixation.

SOXHLET EXTRACTION

Extraction of tissue for 48 hours with absolute alcohol in a soxhlet apparatus removed all Feulgen-positive material except that occurring in nuclei, vascular endothelium, the matrix of cartilage, and elastic tissue. An additional extraction of 48 hours with chloroform, after that with alcohol, caused no further change. Also, both slide and block staining gave similar results after extraction. Sections unhydrolyzed by acid gave a weak but typical Feulgen reaction.

SUMMARY AND DISCUSSION

The experiments on the effect of fixation on subsequent staining show (in agreement with Feulgen-Brauns) that less acid hydrolysis is needed for unfixed tissue in order to prepare it for optimum "nuclear" reaction. In our experiments with block staining, hydrolysis and fixation occurred concomitantly, where as in Feulgen-Brauns' work unfixed dried blood smears were hydrolyzed with HCl.

When fixation, hydrolysis and staining proceeded simultaneously, typical nuclear staining was rather weak. It may be that the excess of sulfite is responsible for the poor results. Our experiments do not necessarily exclude the possibility of making a one-solution fixing and staining reagent based on the Feulgen reaction.

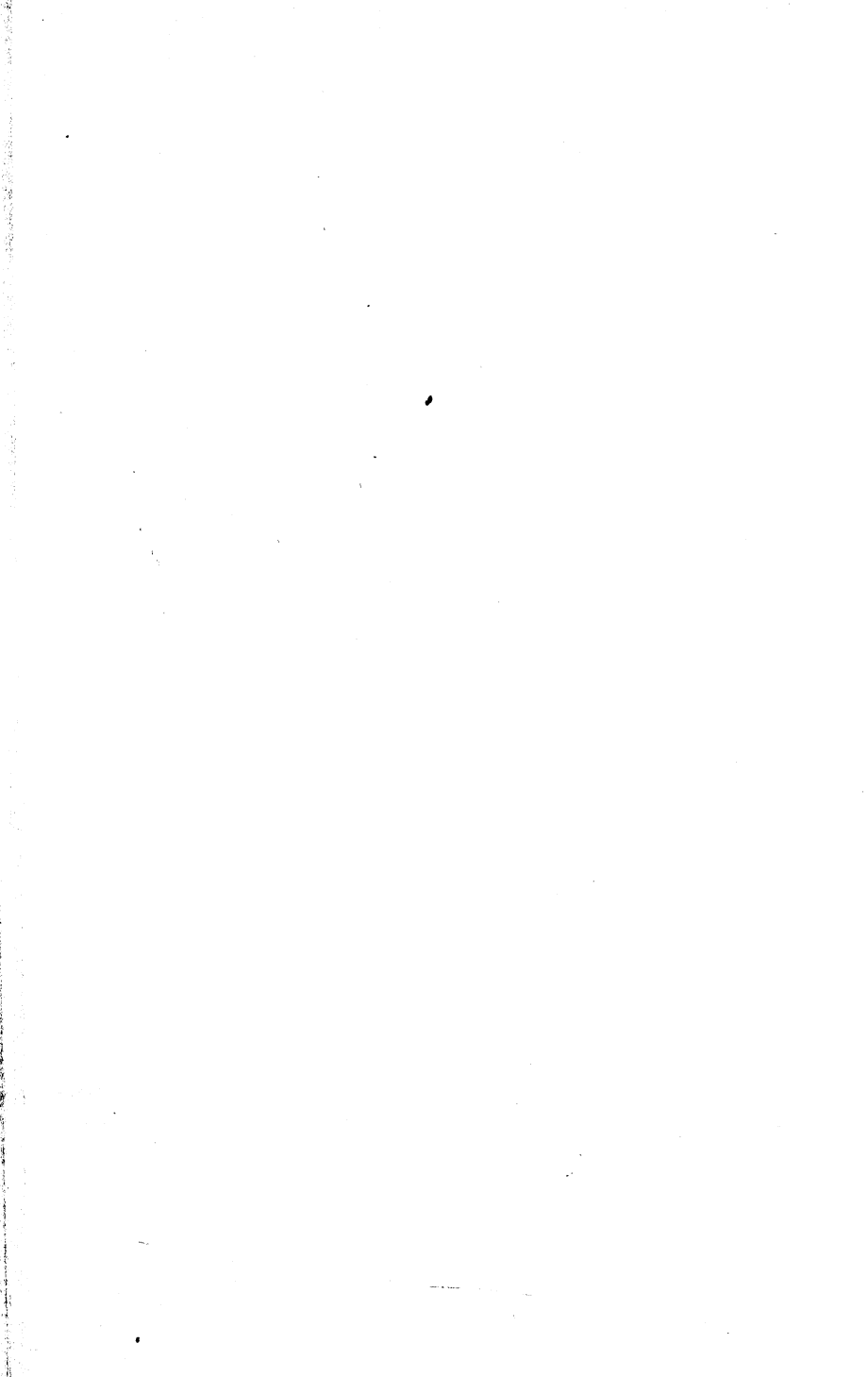
The regular section method is more specific for nuclei than the block method described in our preceding paper. Soxhlet extraction prior to staining in the block shows that most of the non-specific substances are extracted by alcohol. This is similar to the extraction occurring during dehydration and embedding prior to staining on the slide. There are a few non-nuclear components, which are not known to contain thymonucleic acid, such as vascular endothelium, cartilaginous matrix, and elastic tissue, which are likely to remain Feulgen positive even after a very thorough extraction of lipids.

ACKNOWLEDGMENT

This investigation was supported by a grant received from the Biological Stain Commission.

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LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

DYES AND THEIR BIOLOGICAL USES

FURMAN, N. H., and STONE, K. G. A polarographic study of certain anthraquinones. *J. Amer. Chem. Soc.*, **70**, 3055-61. 1948.

Antraquinone and its derivatives that possess the quinone structure give an early polarographic reduction wave that is usually in the neighborhood of -0.6 to -0.7 volt relative to the saturated calomel electrode. Comparison of polarographic $E_{1/2}$ values with potentiometric data indicates that the quinone nucleus is first reduced to the ionized hydroquinone nucleus at the mercury surface even in acidic buffers. Waves of unusual slope are formed in acidic media when there is a sulfonic acid group in the 1- or in both 1- and 5-position. Quantitative estimation of anthraquinones by the polarographic method is generally possible.—R. T. Whittenberger.

MERRILL, R. C., SPENCER, R. W., and GETTY, R. The effect of sodium silicates on the absorption spectra of some dyes. *J. Amer. Chem. Soc.*, **70**, 2460-4. 1948.

Addition of various sodium silicates (colloidal electrolytes) to a solution of toluidine blue O reduced the intensity of the absorption band and shifted the band maxima from $620\text{ m}\mu$ to about $540\text{ m}\mu$, causing a visual change from blue to purple. Silicates shifted the band maxima of rhodamine 6 G about $6\text{ m}\mu$ toward the violet but did not alter the intensity by more than 2%. This dye was orange and fluorescent in water and red and non-fluorescent in silicate solutions. Addition of silicates to a solution of pinacyanol chloride changed the visual color from blue to purple and changed the absorption spectra by decreasing the intensity of the α and β bands to different extents, and by forming new α' and γ bands. The effects of silicates were attributed to sorption and electrostatic interaction of the dye ion with the silicate ions and micelles.—R. T. Whittenberger.

MORRIS, R. J., and BRODE, W. R. The relation between the absorption spectra and the chemical composition of dyes. XX. Induced non-coplanarity in symmetrical benzidine dyes. *J. Amer. Chem. Soc.*, **70**, 2485-8. 1948.

Data indicate that compounds with restricted rotation differ markedly in their absorption spectra from those capable of free rotation. The partial conjugation present in the symmetrical benzidine dyes is aided by the establishment of a more coplanar configuration for the benzidine nucleus. This molecular conjugation is progressively inhibited by the introduction of the relatively large, unreactive methyl groups in the 2,2'- and 6,6'-positions on the benzidine nucleus. For dyes prepared from the tetramethyl substituted nucleus, the insulation to molecular conjugation at the 1,1'-bond appears almost complete.—R. T. Whittenberger.

ANIMAL MICROTECHNIC

DALAND, GENEVA A., and CASTLE, WILLIAM B. A simple and rapid method for demonstrating sickling of the red blood cells: the use of reducing agents. *J. Lab. & Clin. Med.*, **33**, 1082-7. 1948.

The procedure first used by the author was as follows: place a small drop of the blood to be tested on a slide; add 2 drops of a 2% aqueous solution of the ascorbic acid preparation, Cevalin, patented by Eli Lilly and Co., Indianapolis [ascorbic acid 11.0%, anhydrous sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) 0.55%]; mix and press down with a cover slip. Sickling usually appeared within 1 hour. It was later found that a 2% solution of acid sodium sulfite (NaHS_3) could be used

instead of Cevalin and that sickling usually appeared within 15 minutes.—*John T. Myers.*

TOMPKINS, EDNA H. M. Methods to increase accuracy in the use of Hayem's solution for red blood counts. *J. Lab. & Clin. Med.*, **33**, 1180-8. 1948.

The HgCl_2 present in Hayem's solution has a tendency to clump red cells, thus introducing an error. This error may be eliminated by reducing the amount of HgCl_2 from 0.25% to 0.05% and buffering to a pH of 5.7 (Jørgensen's solution), or still better by adding 0.01% of gelatin to Hayem's solution.—*John T. Myers.*

PLANT MICROTÉCHNIQUE

KINSINGER, W. G., and HOCK, C. W. Electron microscopical studies of natural cellulose fibers. *Ind. Eng. Chem.*, **40**, 1711-6. 1948.

Three techniques are described. For metallic shadow casting, the fibers are beaten in water in a Waring Blender for 15-20 min. Small drops of the supernatant slurry are placed on a glass slide coated with a thin film of 0.3% Parlodion in amyl acetate. After drying, the specimen is shadowed either with chromium or gold at an angle of 45° or higher. This is done by evaporating the metal from a small source in a high vacuum onto the specimen. After shadowing, the film is floated off the slide onto water, and mounted in the usual way. In making surface replicas, a film of 3% Parlodion in amyl acetate is cast on a glass slide. Before the film is completely dry, several individual unbeaten fibers are pressed gently against it. The fibers are stripped off with tweezers, and an imprint of their surface is left in the film. The film then is shadowed with chromium at an angle of 15°, and is prepared for examination as before. The electron "stains" used included HgCl_2 , lead acetate, AgNO_3 , CuSO_4 , uranyl acetate, thallium hydroxide, phosphotungstic acid, I-KI, and ZnCl_2 -I. These were applied as 1 to 20% aqueous solutions for an average of 20 to 30 hours to a slurry of the beaten fibers. Fibers were washed by centrifuging several times with distilled water and then mounted on a Parlodion film on the specimen screen. The most satisfactory results were achieved with solutions of lead acetate.—*R. T. Whittenberger.*

MICROÖRGANISMS

DELAMATER, EDWARD D. Basic fuchsin as a nuclear stain for fungi. *Mycologia*, **40**, 423-9. 1948.

Because of unsatisfactory Feulgen reactions in certain pathogenic fungi, the author proposes modifications in the use of basic fuchsin.

Procedure 1. (A) Fix cells in Schaudinn's solution for 1 hr. (B) Wash 15 min. in each of 30%, 20%, and 10% alcohol. (C) Wash in distilled water. (D) Hydrolyze in normal HCl at 60°C., for 10 min. (Optimum period must be determined for each organism, and temperature must be accurately controlled.) (E) Wash in distilled water, 5 min. (F) Stain 5-15 min. in 0.25% aqueous basic fuchsin. (G) Wash in distilled water. This method gives a precise, delicate, and intense nuclear stain, but water mounts fade rapidly; mounts in a glycerin-sugar-gum-arabic mixture will last for several weeks.

Procedure 2. The aqueous basic fuchsin stain can be made permanent by using formalin as a mordant. Proceed as above through steps A-E, then mordant the hydrolyzed cells in 2% formalin for 2-4 min. before exposing them to the stain. Stain for 15 min. in 0.5% aqueous basic fuchsin in 0.04 N HCl. Destaining is controlled by the speed with which the cells are run up through the graded alcohols. After this dehydration, clear in xylene and mount in balsam or Clarite.

Procedure 3. This method combines mordant and stain, so that the resultant solution is 0.5% basic fuchsin, 2% formalin in 0.04 N HCl. Although it eliminates the individual mordanting process, precipitates are more likely to form and the method is more difficult to control than is the case in Procedure 2.—*Elbert C. Cole.*

HISTOCHEMISTRY

ENGSTRÖM, A. Quantitative cytochemical determination of nitrogen by X-ray absorption spectrography. *Biochimica et Biophysica Acta*, **1**, 428-33. 1947.

The theoretical basis for determining nitrogen in biological material by X-ray absorption spectrography is discussed. Using the K-absorption edge of nitrogen

(31.1 Å wavelength), it is possible to determine amounts of nitrogen of the order of 10^{-10} to 10^{-12} g. in sections of tissue 2 to 10 μ thick within an area of 10 μ square. — R. E. Stowell.

ENGSTRÖM, A. **Quantitative micro- and histochemical elementary analysis by Roentgen absorption spectrography.** *Acta Radiol., Suppl.*, 16, 1-103. 1946.

A quantitative roentgen microspectrographic procedure is described which permits analysis of chemical elements with low atomic numbers in single mammalian cells. The method measures the absorption of monochromatic roentgen radiation within microscopic areas of tissue. Either an ionometrical or photographic-photometrical measuring procedure is repeated in a series of wavelengths lying on each side of a long wave roentgen ray absorption edge of the sought-for element. From such data, the quantity of the element in question can be calculated.

The possibilities of quantitative elementary analysis of different tissues are discussed. It is possible to determine phosphorus and calcium in a bony tissue 10 μ square by 10 μ thick or nitrogen and oxygen within a 50 to 100 square μ area, several μ in thickness. The amounts measurable are of the order of 10^{-9} to 10^{-12} grams. The analyses are accurate to within 5-10% and results can be directly correlated with the cytologic picture.

Although such technical methods are too complicated for most biologists, the abstractor believes that with anticipated refinements such methods may become a most important technic for studying the chemical structure of morphologically intact cells. — R. E. Stowell.

ENGSTRÖM, A. **Ultramicroanalysis by X-ray Absorption Spectrography.** *Trans. Instruments and Measurements Conf., Stockholm*, 1947, pp. 71-73.

The basis and equipment for X-ray absorption spectrography is presented. In addition to biological uses, applications to metallic ions as zinc and alloys of Al, Zn, Cu and Fe are given. — R. E. Stowell.

ENGSTRÖM, A., and JAKUS, M. A. **Intracellular determination of protein by X-ray microspectrography.** *Nature*, 161, 168. 1948.

The measurement by X-ray microspectrography of protein bound to phosphotungstic acid is discussed. Microradiograms of ganglion cells treated with phosphotungstic acid at magnifications of 130 and 260 times show more phosphotungstic acid bound within the cytoplasm than within the nucleus. It is properly emphasized that precautions must be taken before such data are translated into concentrations of intracellular protein. — R. E. Stowell.

ENGSTRÖM, A., and LINDSTRÖM, B. **Histochemical analysis by X-rays of long wavelengths.** *Experientia*, 3, 191. 1947.

The use of long wavelength filtered primary radiation in special equipment for microradiography is described. When secondary radiation from sulphur and chlorine with K-radiation 5.36 and 4.72 Å respectively is employed, the sulphur content in different parts of a section of skin can be determined. The amount of sulphur is obtained in 10^{-10} g. for a measurement surface of 1000 sq. μ . By allowing heavy elements as iodine, lanthanum, and tungsten to combine with other substances such as glycogen, nucleic acids and proteins, one can produce an "X-ray staining" to make histo- and cytochemical observations. — R. E. Stowell.

SERRA, J. A., and QUEIROZ-LOPES, A. **Direct demonstration of basic protein in the chromosome and nucleolus.** *Naturwissenschaften*, 32, 47. 1944.

The authors applied α -naphthol-hypobromite to demonstrate the arginine-rich basic protein in the nucleolus and chromosome of *Chironomus* and two plant roots, *Vicia faba* and *Allium cepa*. They found that the salivary gland chromosome shows intensive reaction in its band, while its interband shows a weak reaction. The former are probably composed of a histone-type of protein and the latter probably of globulin-like protein. Both the nucleolus and chromosome contains a large amount of basic protein and the chromosome shows increasing intensity of arginine reaction during mitosis. The distribution generally corresponds to that of thymonucleic acid, but after removal of the nucleic acid by nuclease, the chromosome and nucleolus show the same intense reaction. — C. S. Lee.

SERRA, J. A., and QUIEROZ-LOPES, A. **Données pour une cytophysiologie du nucléole. I. L'activité nucléolaire pendant la croissance de l'oocyte chez des Helicidae.** *Portugal. Acta Biol.*, 1, 51-94. 1945.

Observations were made *in vivo* and on fixed tissues from the ovotestis of two species of pulmonate land snails, *Helix aspersa* Müll and *Tachea nemoralis* L. The *in vivo* observations were made in hanging drop preparations in Ringer's solution and in saline of five times the normal concentration of salt. The fixatives used were Carnoy's acetic-alcohol, Serra's acetic-formol-alcohol and 10% formalin. The histological observations were made on sections stained with iron hematoxylin and eosin and with pyronine-methyl-green. Histochemical observations were made by the use of: (a) the Feulgen stain; (b) reaction for nucleic acid phosphorus (Portugal. Acta Biol, 1, 111-22, 1945); (c) intensity of staining with acid and basic dyes in solution with reduced surface tension; (d) determination of isoelectric point; (e) staining of lipids; (f) reactions for arginine, —SH groups and tryptophane, (g) modified Millon reaction; (h) ninhydrin reaction; (i) digestion of sections with nucleases.

The authors are of the opinion that their observations demonstrate that the nucleolus is active in the synthesis of cellular substances, particularly ribonucleoprotein. During the period of most rapid growth of the oocyte, the nucleolus grows even more rapidly. It appears to give rise to daughter nucleoli by budding, and at the same time nucleolar inclusions rich in basic proteins are seen. These daughter nucleoli appear to dissolve in the nuclear sap, and their extrusion into the cytoplasm has not been observed. During the period of rapid growth the concentration of ribonucleoprotein in the nucleolus appears to decrease, and that in the cytoplasm increases, initially most prominent about the nuclear membrane. Heterochromatin appears to be of little importance in the growth or elaboration of cytoplasm of the oocyte. The authors believe that the fundamental role of the nucleolus of the oocyte during its period of rapid growth is the elaboration of nucleoprotein which in turn serves as a sort of "nucleus" or point of departure for all protein synthesis.—E. B. Taft.

SERRA, J. A. and QUEIROZ-LOPES, A. **Une méthode pour la démonstration histochimique du phosphore des acides nucléiques.** *Portugal. Acta Biol.*, 1, 111-22. 1945.

Because of dissatisfaction with the previously described methods for demonstration of the phosphorus of nucleic acids, the following method was elaborated:

Fixation: Small pieces of tissue are fixed in the alcohol-formalin-acetic-acid fixative devised by Serra. After fixation tissues are washed in running water and then distilled water.

Reagents: (1) Dissolve 0.5 g. ammonium molybdate in 20 ml. of distilled water. Add 10 ml. of concentrated HCl (30%). Dilute to a volume of 50 ml. with distilled water. (2) Dissolve 25 mg. benzidine in 5 ml. of 98-100% pure acetic acid. Dilute to 50 ml. with distilled water. (3) Saturated solution of sodium acetate.

Procedure: (1) Animal or vegetable tissues are placed in stoppered tubes containing 2-3 ml. of reagent 1 and incubated at 10-12° C. for a minimum of 2-3 weeks. (2) The tubes are then placed in another bath at 20-25° C. for 2-3 days. (3) Sections from reagent 1 are placed on slides or in small dishes with a drop of the reagent from the tube in which they were incubated. (4) One drop of reagent 2 is added and allowed to react for approximately 3 min. (5) Then two drops of reagent 3 are added (an excess is not harmful). The sections rapidly turn an intense blue. (6) Microscopic observation may be made in the acetate solution or better in glycerin. In glycerin, the color fades somewhat. For best results add several crystals of sodium acetate to the glycerin. For a semi-permanent preparation the coverslip may be sealed with the lanolin-colophonium cement of Romeis.

Results: The sites of hydrolysis of organically combined phosphorus are stained varying intensities of blue by this method. The nucleus and nucleolus are particularly well demonstrated as are masses of ribonucleoprotein in the cytoplasm. The reaction must be controlled by the use of ribonuclease (the authors used that obtained from rice hulls) since phospholipids and phosphoprotein compounds give a similar color. Comparison with sections stained by the Feulgen reaction is also essential.—E. B. Taft.

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PLASTIC EMBEDDING OF THICK CELLOIDIN SECTIONS OF NERVE TISSUE

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ABSTRACT.—A method is described for mounting Golgi-impregnated and Weigert-stained thick celloidin sections of brain and spinal cord in transparent plastic. Finished mounts have good optical properties and are suitable for macroscopic and microscopic observation. The durability of such preparations makes them superior to similar material prepared by the more conventional methods. Holes of suitable size were cut in matrices of $2.5 \times 5 \times \frac{3}{16}$ inches Plexiglas. Ward's Bio-plastic was used to form a base for the holes and also as the embedding medium for the sections. Plate glass formed a working substrate and gave a polished surface to the plastic base and later to the top of the preparation. For Golgi material (200μ) the celloidin was removed by dioxane. A dioxane-plastic bath preceded plastic embedding. For Weigert material ($30-40\mu$) celloidin was not removed due to fragility of sections. Prior to plastic embedding, they were subjected first to benzol and then to a benzol-plastic bath.

The superb preparations of anatomical specimens in transparent plastic demonstrated by Kampmeier and Haviland at the A.A.A.S. meetings at Chicago in 1947, followed by publication of their methods and a good review of the field (1948), have been a stimulus to further work along these lines. In addition to mounts of gross specimens, these workers have briefly described mounting of large thick celloidin sections. In this paper we describe methods which have given us the best results with thick celloidin sections of cat and rabbit brain and spinal cord following the Golgi and Weigert technics. Completed mounts are durable, have good optical properties and may be observed with the 16 mm. lens of the compound microscope. The Golgi preparations are superior to those mounted by the balsam method.

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By our method a sheet of Plexiglas of 3/16 inch thickness is sawed into rectangles 2½ by 5 inches, and two smooth-sided 2-inch holes are machined into each rectangle. This diameter easily accommodates transverse sections of cat brain. It is advisable to use a hole with a diameter somewhat larger than the specimen, in order to decrease curvature formed by a meniscus when plastic is poured. The size may be altered to fit other materials.

A pool of Ward's Bio-plastic plus 18 to 20 drops of catalyst per 100 ml. of plastic is poured onto a piece of clean plate glass (Fig. 1). The cell is placed upon it (Fig. 2,3), and a 24-hour room temperature hardening period is allowed. The cell is then filled with more plastic to the level desired for the section (Fig. 4). After pouring the additional plastic into the cell, the preparation is placed in a dust-free, level place to harden, first for 24 hours at room temperature, then 36 hours at 37°C., and finally at 55°C. for 12-16 hours. It is cooled slowly. The rectangles usually free themselves spontaneously from the glass base leaving a polished surface, which eventually becomes the bottom of the finished preparation. Too rapid cooling may cause the glass to shatter. In our experience plate glass is superior to ordinary window glass as a base, since the plastic frees itself more easily from it with no shattering of the glass. If the plastic does not spontaneously pull away when thoroughly cooled, the seal may be broken by lightly scoring the edge of the cell several times with a sharp, sturdy scalpel (Fig. 7) and then slightly lifting the corner with a sharp razor blade (Fig. 8).

By this procedure, excess plastic from around the edges of the mount will be left on the glass. Having been exposed to air during the curing period it will not be completely polymerized. It may be removed from the glass by gentle scrubbing with dioxane-soaked cotton. Thoroughly hardened plastic polymerized in the absence of air is little affected by dioxane, however, and must be carefully scraped off with a sharp razor blade.

PLATE I

A pictorial description of a method for mounting thick celloidin sections in plastic. Curing (polymerization) of the plastic occurs between Fig. 3 and 4, 4 and 5, 6 and 7 and between 10 and 11.

1-4 Preparation of a plastic base for pre-cut Plexiglas cells, using plate glass as a working substrate.

5 Floating sections onto the prepared base.

6 Pouring an additional thin layer of plastic to cover the sections and fill the cell.

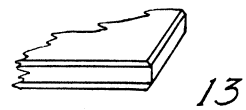
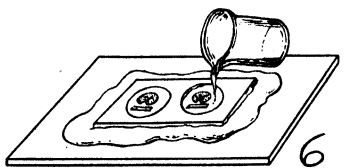
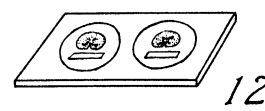
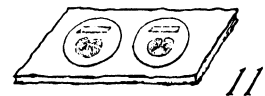
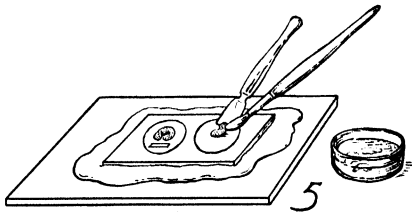
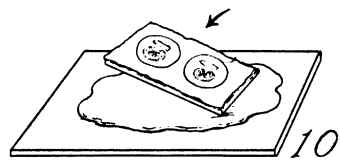
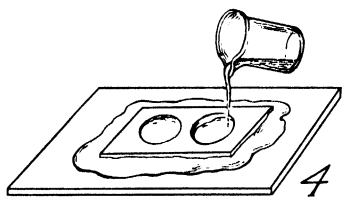
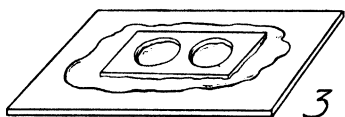
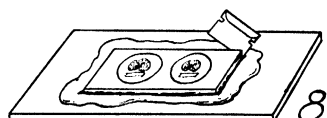
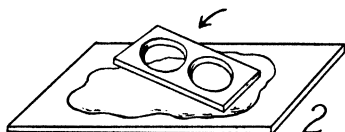
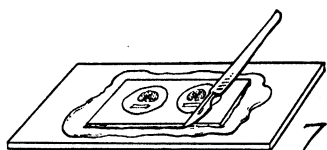
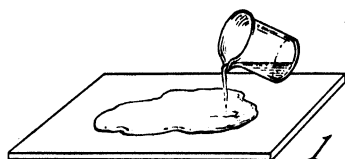
7-9 Removing preparation from the glass.

10 Inversion onto new pool of plastic to finish the top surface.

11 Rough-edged preparation ready for finishing.

12 Excess plastic has been removed by band-sawing or sanding.

13 Beveling of the edges is not necessary but adds to the appearance of the preparation.



GOLGI-STAINED MATERIAL

An outline for the procedure for material stained by the Golgi technic is as follows:

1. Remove celloidin-embedded blocks from chloroform hardener, and section at $200\ \mu$ on the sliding microtome, keeping the block and the knife flooded with 80% alcohol.

2. Transfer sections, as cut, to 95% ethyl alcohol, in which they may remain for not more than five minutes. Cut only a few sections at one time, in order that the process may continue to the next step without undue prolongation in the 95% alcohol.

3. Place the sections in equal parts 95% ethyl alcohol and dioxane until the celloidin is dissolved.

4. Transfer to pure dioxane for two minutes.

5. Use a second pure dioxane for two to five minutes to insure complete dehydration. (Slight traces of water carried into the plastic will cloud the mount badly.)

6. Place in a mixture of one-part dioxane and two parts of the uncatalyzed plastic, until the sections become well-cleared. Use an air-tight bottle, either a screw cap or rubber-sealed jar, since the mixture of dioxane and plastic (or benzol-plastic mixtures) harden in a few hours at room temperature if exposed to air. Furthermore, sections become opaque if allowed to stand in an exposed mixture. Storing in the refrigerator at 5° to 7°C . prolongs the time the mixture will remain fluid. This phase of the procedure is of practical importance, since Golgi-stained specimens to be mounted in balsam or clarite must be carried to completion in the shortest time possible. By the plastic method the tissues may be run up to the dioxane and plastic as rapidly as possible and may then be stored for future mounting. If the plastic begins to set, however, failure will ensue; no solvent has been found by which it can be reconverted to a solution. Benzol-plastic mixture will give the same results if left exposed.

7. To mount the section, float it onto a spatula and carefully slide it into the previously-hardened base in the cell (Fig. 5). (Printed labels which have been soaked for ten minutes in a plastic-dioxane mixture may also be put into the cell at this time.) Fill the cell with catalyzed plastic (Fig. 6), and set it in a *dust-free*, level place to cure for 24 hours at room temperature.

8. To finish the preparation, pour a pool of plastic plus catalyst onto the surface of the glass, as in Fig. 1, and lower the unfinished side of the cell onto the mixture (Fig. 10). Allow sufficient plastic to flow out from the edges of the cell so that they are well sealed.

Finishing time can be sped up to overnight at room temperature followed by 24 hours at 37°C. and finally 12 hours at 55°C.

WEIGERT-STAINED MATERIAL

For the Weigert stain, material is sectioned at 30 to 40 μ . Benzol is used as the final bath prior to plastic, since benzol does not dissolve celloidin. This is necessary with such thinner sections which cannot be handled without a celloidin matrix. Subsequent to staining, sections are run up to 95% alcohol, then into carbo-xylol to complete dehydration and for clearing. Sections left in carbo-xylol for 5-10 minutes harden somewhat and can be handled more easily. They are placed in pure benzol for 5-10 minutes to wash out the carbo-xylol. They can remain in the benzol overnight without damage. The depression in the prepared base is flooded with benzol, and the section is transferred quickly by means of a spatula and a soft brush (Fig. 5). A pipette filled with benzol can be used to keep the spatula flooded. Excess benzol is removed from the depression with a fine pipette, and plastic is carefully brushed onto the section to prevent drying. The cell is then completely filled with plastic and the curing process as described for the Golgi material is followed.

Rough edges of the preparation (Fig. 11) are dressed by using a hand-saw to remove excess plastic. The edge is smoothed (Fig. 12) with a fine file. Addition of a slight bevel (Fig. 13) to the edge improves the appearance of the specimen. It is unnecessary to polish the sides, since the specimen is not viewed from these surfaces.

DISCUSSION

It cannot be said from our results that plastic is applicable to general histological technic, since it has here been used for silver-impregnated (Golgi) and hematoxylin-stained (Weigert) specimens only. In both cases the method has been limited to thick celloidin sections. Attempts to shorten the method by combining the steps of embedding and surfacing into a single operation proved to be unsatisfactory.

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ACID THIONIN STAIN FOR NISSL BODIES ON FROZEN SECTIONS

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ABSTRACT.—Using a buffered acid thionin stain with carbol-xylene as a clearing agent, a reliable stain for Nissl bodies may be performed on frozen sections of fresh or old formalin-fixed material in a relatively short period of time. The technic is simple: the buffering of thionin makes regressive differentiation unnecessary.

A reliable, accelerated stain for Nissl bodies in frozen sections of fresh or aged formalin-fixed tissues has been evolved. The use of buffered thionin was derived almost directly from our current technic for the staining of Nissl Bodies in paraffin sections (1) which in turn resembled closely Windle's buffered thionin (2).

To find the optimum pH level, sections from human and animal tissues were stained at different pH levels. At pH 3.5, the immediate results were satisfactory but the stains faded after a few days exposure to light. The sections stained at pH 5 were too dense for immediate examination. At pH 4 the Nissl bodies remained distinct with a slight fading of glial cell nuclei after 80 days exposure to a 60 Watt light bulb at about 20–50 cm. distance.

The stock solution of 0.1% thionin in aqueous M/100 acetate buffer of pH 4 has been kept at room temperatures of 22–35°C. for 1 year without impairment of staining qualities or appreciable shift in pH level. The solution was kept tightly stoppered and the small quantities required for use were poured out, avoiding insertion of pipettes. Acetate buffer was used because it was found that sodium acid phosphate precipitated thionin.

Heretofore, the use of carbol-xylene after basic aniline dyes has been avoided because of its fading properties. In this method 1:3 carbol-xylene extracts the stain slightly, and with a 1:4 proportion and a few seconds immersion of the section, only a negligible amount of stain is removed. In a few cases of overstaining longer immersion will remove the excess of thionin.

The sections cleared in Weigert's 1:3 carbol-xylene and control sections cleared in pure xylene were exposed to a 60 W. light bulb for 80 days. The Nissl bodies remained distinct in most instances, with slight fading of glial cell nuclei. Clearing in carbol-xylene renders the sections pliable, flat and free of wrinkles, and facilitates

mounting. After xylene clearing, sections are brittle, wavy and wrinkled, and mounting is difficult.

Three mounting media were tested for stain permanence after clearing with pure xylene and with carbol-xylene. After 80 days exposure to light, sections mounted in polystyrene and in Hartman Leddons No. 7885 resin, with a few exceptions, presented sharply stained tigroid. With Canada balsam as the mounting medium, the sections cleared in pure xylene remained fairly satisfactory, but all of those mounted in Canada balsam after clearing in carbol-xylene faded in 1-2 days.

THE TECHNIC

Stock thionin solution:

Thionin (CC) (C.I.No. 920)	400 mg.
M/5 acetic acid solution.	16 ml.
M/5 sodium acetate solution.	4 ml.
Distilled water.	380 ml.

Use tissues fixed in aqueous 10% formalin. Place in watch glasses or small Stender jars with covers.

1. Freeze and cut sections 10-15 μ .
2. Transfer to distilled water.
3. Transfer to 70% ethyl alcohol 5 min. (old formalin material especially).
4. Wash in distilled water.
5. Stain 10 seconds in 1-2 ml. of the 0.1% aqueous thionin in pH 4 acetate buffer.
6. Wash in distilled water.
7. Dehydrate in 95% and 100% ethyl alcohol or 2 changes of acetone.
8. Clear for few seconds in 1:4 carbol-xylene.
9. Place on slide, blot, mount in polystyrene or Hartman Leddons No. 7885 resin.

RESULTS

Deep blue Nissl bodies and glial nuclei on pale or colorless background with both fresh and old formalin-fixed material. If there is too blue a background, this can be corrected by exposure for a few hours to sunlight or a lamp bulb.

Acknowledgment is made to Dr. R. D. Lillie for advice and criticism.

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A NEW FIXATIVE FOR VAGINAL SMEARS

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ABSTRACT.—Two vaginal smear fixatives have been presented for use in cytologic studies by the Papanicolaou technic for the diagnosis of cancer of the genital tract. They are to be used in lieu of equal parts of ethyl alcohol and ether, because of the volatility, waste through evaporation, fire hazard, and expense. They are (a) tertiary butyl alcohol, 75% and ethyl alcohol, 25% (b) tertiary butyl alcohol, 75% and ethyl phosphate, 25% (by volume). The cytologic details and staining qualities of vaginal smears have been maintained or improved.

Since the introduction of the Papanicolaou technic of vaginal smears for ovarian endocrine studies (1933) and the cytologic detection of cancer (1945), the fixative of choice has been equal parts of ethyl alcohol and ethyl ether.

There have been several disadvantages to this mixture, namely, the expense of the ethyl alcohol to smaller laboratories and physicians who cannot obtain it tax free; the marked volatility of the mixture which during the summer months and in warm climates makes for waste and causes ether fumes (the boiling point of ether being 34.6°C.); the handling of the ether during the preparation of the mixture produces the possibility of an explosive atmosphere, especially if the laboratory is not well ventilated.

For these reasons a new fixative was sought. A fixative that would preserve the cytologic details, that would not produce shrinkage of the cells or alteration of the staining qualities; and that would be more stable, less expensive, and easily available.

Johansen (1935) first introduced tertiary butyl alcohol as a dehydrating agent for tissues. During the recent war, because of priorities on ethyl alcohol for civilian use, Stowell (1942a and b) reintroduced it in microtechnic. He found it unusually satisfactory for the dehydration of mammalian tissues. He states "it causes less shrinkage than dioxane, xylene, or chloroform". The cytologic details are well preserved.

Tertiary butyl alcohol [$(\text{CH}_3)_3\text{COH}$], has a molecular weight of 74.12. Below 25.5°C., the melting point, it appears as rhombic prisms

or plates. Above this temperature it is a clear, colorless liquid with a slight odor. The boiling point is 82.8°C., the index of refraction is 1.38779, the density 0.7887 at 20°C. It is soluble in water, ethyl alcohol and ether in all proportions.

Stowell (1942, a) uses tertiary butyl alcohol in conjunction with ethyl alcohol—a 100% solution containing 75ml. tertiary butyl alcohol and 25 ml. of absolute ethyl alcohol. This combination is an excellent fixative for vaginal smears. It has been used in this laboratory for the past year with gratifying results. Excellent cytological detail is maintained with little or no shrinkage. The staining characteristics using the Papanicolaou stain are maintained or slightly improved.

To completely eliminate the ethyl alcohol, a new fixative and dehydrating agent was substituted, namely, triethyl phosphate ($(C_2H_5O)_3 PO_4$). This reagent was introduced by Nelson (1945). It displaces water in tissues with slight if any shrinkage or distortion.

The physical properties of triethyl phosphate (commonly called ethyl phosphate) are, molecular weight 182.16; boiling point 218°C. It is a clear, colorless liquid, with a very slight pleasantly sweet odor. It is soluble in water, ether, ethyl and tertiary butyl alcohols and xylene. Index of refraction is 1.40616 at 10°C. Density is 1.0686 g./ml. at 25°C.

For the past year, the combination of tertiary butyl alcohol, 75%, and ethyl phosphate, 25%, has been used extensively in this laboratory for vaginal smear preparations with excellent results.

When slides are removed from this fixative it is not necessary to work rapidly to prevent the slide from drying, as there is sufficient ethyl phosphate covering the smear after the tertiary butyl alcohol evaporates, due to the high boiling point of the ethyl phosphate.

The smears are fixed in this fixative within 5 minutes and they have been left in the fixative for over 3 months without alteration in the cytologic details or staining qualities.

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A CHEMICAL AND HISTOCHEMICAL STUDY OF THE TECHNIC FOR ACID PHOSPHATASE¹

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ABSTRACT.—A chemical and histochemical study of Gomori's acid phosphatase technic showed that the causes of its unreliability were: (1) that fixation and other steps of histological procedure inactivate the enzyme to a great extent; (2) the enzyme may diffuse, as demonstrated in frozen sections of acetone-fixed material; and (3) some absorption of lead by the sections takes place. Much of this unreliability is avoided, however, by maintaining as low a temperature as possible during fixation and dehydration, with exposure to the temperature of the paraffin oven for the shortest possible length of time. The relative insolubility and thermo-stability of the enzyme, moreover, indicate the possibility of devising a more satisfactory technic in the future.

Ever since 1941, when Gomori described the cytochemical technic for acid phosphatase, a number of reports have appeared in the literature bringing evidence against the specificity and for the unreliability of the method. These papers have pointed out, among other things, the lack of correspondence between chemical data and cytochemical results (Heinzen, 1947), the resistance of the enzyme to inactivation, the non-specific reaction with ascorbic acid (Lassek, 1947), the reaction in injured nerves after probable destruction of enzymatic activity, and the extraction of the enzyme by water, judged cytochemically (Bartelmez and Bensley, 1947). All this work was done on nerve tissue. Stafford and Atkinson (1948) reported chemical data on the destruction of the enzyme by alcohol and acetone fixation and by paraffin embedding.

The authors have made an attempt to study the factors involved in the unreliability and in the so-called non-specificity of the method. It seemed that the unreliability could be the result of two phenomena that can take place during the histological procedure, to wit, inactivation of the enzyme and its extraction from the tissue.

The so-called non-specific reaction could be attributed to incomplete inactivation of the enzyme or to adsorption of lead on histologi-

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cal structures or both. It is interesting to note that practically all reports on false acid phosphatase reactions are on nerve tissue which has an unusual affinity for heavy metals.

We wish to report here results on the action of fixatives, histological procedures and various agents upon acid phosphatase activity, as judged by chemical and cytochemical methods.

MATERIAL AND METHODS

Adult rat and mouse liver was used in this investigation. Liver tissue was chosen because of its high enzyme content and the structural homogeneity of the organ. Animals of both sexes were used, since sex does not influence the enzyme content of the liver (Kochakian, 1947). The animals were killed by a blow on the head, the abdominal cavity opened, fragments of the liver removed and weighed on a torsion balance. Preliminary results showed marked uniformity in the enzyme content of 80 to 120 mg. fragments from 5 different portions of the liver.

Chemical method. Acid phosphatase activity was determined in aqueous extracts (20 to 40 mg. of tissue per ml.) made according to Greenstein (1942). When homogenates were used, 0.5 ml. of a 10% Potter-Elvehjem homogenate was blown into 10 ml. of chilled fixative. It was usually kept in the ice box for 3 hours, then centrifuged, and the precipitate, re-homogenized in saline, used for assay. If the tissue had been previously subjected to the action of fixatives or to histological procedures, enough water was added to make up to their initial weight. The King-Armstrong method as described by Gutman and Gutman (1940) was used. In the majority of the cases incubation time was one hour but when the main interest was the demonstration of the presence of small amounts of enzyme, the incubation period was extended to 12 hours. The effect of dilution of the extract on its activity showed no linear proportionality when activities of 1:2, 1:5, 1:10, 1:20 and 1:50 dilutions were determined. The activity was slightly higher than expected in the higher dilutions. Therefore, the percentages referred to in this paper are only approximate. This is to be borne in mind especially in the case of considerable inactivation in tissue blocks subjected to histological procedures.

Cytochemical method. Frozen sections were used in view of the constant results obtained by the acid phosphatase technic when applied to frozen material. Before sectioning, the blocks were kept in water for 10 minutes. Frozen sections 20 to 40 μ thick were incubated in the following mixture, cleared by filtration before use: dis-

tilled water, 10 vol.; N/10 acetate buffer pH 5, 6 vol.; 2% sodium glycerophosphate (50% alpha, May & Baker), 2 vol.; and 2% lead nitrate, 2 vol. Sections were rinsed in distilled water for 2 to 5 minutes, transferred to a dilute solution of ammonium sulfide, rinsed in water, dehydrated and mounted. Controls were run in a mixture containing no glycerophosphate, and also in the complete incubating mixture to which sodium fluoride was added in a final concentration of N/400.

RESULTS

Effect of fixatives on acid phosphatase. Chemical results obtained with fixed liver blocks are summarized in Table 1.

With homogenates less inactivation was obtained, but even so, fixation in chilled absolute alcohol for 3 hours inactivated 50% of the enzyme, while chilled acetone inactivated about 35%. The effect of dilute chilled alcohol was also studied on homogenates because of the possibility that low concentrations of fixative prevailing during the stage of its gradual penetration into the tissue may be especially harmful. However, homogenates fixed in concentrations of 17, 25, 40, 60 and 100% alcohol were found to be inactivated to about the same extent. On the other hand, the temperature of the fixative seems to be very important, for homogenates fixed at room temperature either in alcohol or in acetone showed an inactivation in excess of 80%.

Phosphatase activity was examined histochemically in 30 μ thick frozen sections of liver blocks fixed in the same fixatives and for the same length of time. Incubation time was 45 and 90 minutes. Alcohol-formol-acetic-acid fixative, alcohol-formol (10:1), sublimate-alcohol and methyl alcohol completely inactivated the enzyme the, color development being equal to that of fluoride control sections. Formol-saline and 80% alcohol resulted in diminished activity as compared with acetone fixation.

Experiments were also made with frozen-dried blocks of liver in the hope that dry dehydration of the tissue would prevent inactivation of the enzyme.

Frozen-dried blocks were fixed overnight at 5°C. in 60, 80 and 100% alcohol and acetone and in formol-alcohol 1:10. They were subsequently treated in the refrigerator with 100% alcohol for 24 hours, alcohol-ether, 2 changes, one hour each, 4% collodion in alcohol-ether overnight, chloroform, 2 changes, one hour each, and 3 hours in paraffin in a 60°C. oven. Sections of these blocks did not stain darker or more uniformly than those of control blocks fixed freshly in chilled acetone.

The effect of the dehydrating and embedding procedure on acid phosphatase. Small blocks fixed for 2 and 24 hours in absolute acetone at 5°C. and subsequently carried through part or all of the embedding routine were used. The experiment is summarized in Table 2. Data on phosphatase activity are averages of 4 blocks each.

The inactivation produced by alcohol at room temperature is so great that it was thought it may overshadow other inactivating agents used later on in the routine. However, the harmful effect of neutral hydrocarbon solvent at the temperature of the paraffin oven could be demonstrated by fixing homogenates in chilled acetone, treating them for 3 hours with xylene at 60°C., washing them with acetone

TABLE 1.—EFFECT OF FIXATIVES ON THE ACID PHOSPHATASE ACTIVITY OF LIVER BLOCKS

Fixative	Time	Residual activity
	(Hours)	%
Acetone (6° C.).....	24	22.0
Acetone (6° C.).....	2	39.0
10% formol-saline (6° C.).....	12	9.1
80% alcohol (6° C.).....	12	11.4
1 part saturated aqueous HgCl ₂ and 1 part absolute alcohol (6° C.).....	12	4.0
Alcohol-formol-acetic acid (8:1:1) (6° C.).....	12	1.5
Methyl alcohol (6° C.).....	12	0.0

TABLE 2.—EFFECT OF DEHYDRATING AND EMBEDDING ON THE ACID PHOSPHATASE CONTENT

Acetone	100% alcohol*	Xylene*	Paraffin 56° C.	Xylene*	100% alcohol*	70% alcohol*	Water*	Residual activity
Hours	Hours	Hours	Hours	Hours	Hours	Hours	Minutes	%
24	—	—	—	—	—	—	—	28
24	2	—	—	—	—	—	—	4
24	2	2	—	—	—	—	—	2
24	2	2	3	—	—	—	—	3
2	2	3	1	1	1	1	30	7
2	—	—	—	—	—	—	—	39

*Room temperature.

and resuspending them in water. Acid phosphatase activity of the heated homogenate was only 40% of that of the unheated control.

Histochemical experiments were performed with small blocks of acetone-fixed material. They were divided in groups and treated as shown in Table 2. Frozen sections of the central portions of 2 blocks of each group were incubated for 45 min. in the substrate. Pronounced inhibition was noted in sections of blocks treated with alcohol, xylene and paraffin as compared with untreated ones.

Extraction of enzyme from tissue by fixatives and by water. It was attempted to demonstrate enzymatic activity in acetone, 80% alcohol and 10% formol-saline, used as fixatives for blocks of tissue weighing about 1 g. Time of fixation was 12 and 24 hours. The volume of the fixatives was reduced to about 6 ml. in a calcium chloride vacuum desiccator, and the phosphatase content was determined, an incubation period of 12 hours being used. The possibility that enzymatic activity in the fixatives may have derived from hemolyzed red blood cells led to the use of saline-perfused livers. This experiment showed that the phosphatase content of the liver tissue itself was not modified by this procedure. No significant activity could be detected in the fixatives. To test the possibility of the extraction of acid phosphatase from sections, fixed small blocks of about 1 g. of liver were cut in 50μ thick frozen sections. The sections were kept in distilled water at 37°C . for 4 to 5 hours. After filtration through gauze, the volume of water was reduced to 6 ml., and the activity of 2 aliquots determined. Amounts representing at most 5% of the total activity of the fixed blocks could be found. No attempt was made to recover the enzyme quantitatively from the sections.

Experiments were also made with homogenates in order to determine the solubility of acid phosphatase after fixation. A certain amount of homogenate fixed in chilled absolute acetone was resuspended in water, rehomogenized and centrifuged at 2000 r.p.m. for 10 minutes. Determinations of acid phosphatase activity were made on the total homogenate, on the washed precipitate and on the supernatant fluid. About 50% of the activity was found in the precipitate and the other 50% in the supernatant fluid.

Frozen sections of tissue fixed in acetone, 80% alcohol and 10% formol-saline were kept in water at 37°C . for 2 to 5 hours. They were then incubated in the substrate and the activity was compared with that of untreated sections. A slight decrease in activity was noted when extraction was maintained for 5 hours. No decrease in activity was seen after 1 or 2 hours.

Effect of temperature on acid phosphatase activity. A few chemical experiments were performed on aqueous extracts of liver. It could be shown that at 61°C . enzymatic activity was about the same as at 37°C . At 82°C . only about 25% of the activity remained.

In histochemical experiments acetone-fixed frozen sections were incubated in the substrate at 6, 37, 61 and 82°C . Activity was distinctly lessened at 6°C . and greatly enhanced at 61°C ., as compared with results obtained at 37°C . At 82°C . a great reduction in activity

was found. In the substrate mixtures incubated at the higher temperatures, a white precipitate was noted at the end of the incubation period. This precipitate was a lead salt (probably phosphate), for it was blackened by sulfide.

Inhibition by sodium fluoride. The amount of sodium fluoride sufficient to produce a significant inhibition was studied with frozen sections of liver blocks fixed in 10% formol-saline; incubation times were 45 and 100 min. Concentrations of sodium fluoride down to 0.02 mg. per 10 ml. of substrate ($M/17,000$) gave a distinct inhibition. On the other hand, sodium fluoride incorporated at a concentration of 2 mg. per 10 ml. in the formol-saline fixative ($M/170$) did not inhibit enzymatic activity of frozen sections.

Effect of various other chemicals. In frozen sections, ferricyanide (0.01M), ascorbic acid (0.04M) did not affect the enzymatic reaction. Manganous sulfate (0.01M) as recommended by Moog (1943) could not be tried under our conditions because it gave a heavy precipitate of lead sulfate when mixed with the substrate. Ammonium hydroxide (5 drops of a concentrated solution in 20 ml. of water) entirely inactivated the enzyme after an exposure of 20 minutes (acetone-fixed frozen sections). Hydrogen peroxide (0.01M) had no influence on the activity of homogenates, while cysteine (0.01M) increased activity by more than 50%. As the Folin-Ciocalteu reagent is reduced by cysteine, activity was determined with a method for phosphorus. Three ml. of a 0.5% aqueous saponin solution added to 10 ml. of substrate had no influence on the color development of frozen sections.

Extraction of lead phosphate. The cytochemical method was followed to the point just before transferring the sections to ammonium sulfide. They were then kept for 2 hours in distilled water. No loss of color development could be observed.

DISCUSSION

Before attempting to correlate chemical and cytochemical data we wish to emphasize that results cannot be compared directly. One reason for this is that different substrates were employed in the chemical and cytochemical methods. Another reason is that the factors controlling the penetration of the substances used in the cytochemical technic are poorly understood. Penetration through cellular structures and various adsorption phenomena can be influenced in unpredictable ways by temperature and chemical agents.

Effect of fixatives. Our chemical data agree in general with those presented by Emmel (1946) and by Stafford and Atkinson (1948) on the marked decrease in enzymatic activity after fixation in formol-

saline, acetone and 80% alcohol. As it was impossible to prove extraction of acid phosphatase by these fixatives, the decrease in activity seems to be due exclusively to inactivation of the enzyme. In the case of formol fixation, *in vitro* results speak in favor of this interpretation (Emmel, 1946; Abul-Fadl, 1947), but for alcohol fixation they are in contradiction with those of the second author. Emmel's figures were higher than ours, possibly because he worked with homogenates which were dialyzed to remove formol. Gomori (1941) showed the marked reduction of enzymatic activity after acetone fixation by comparing the reactions of fixed and unfixed tissue. The higher enzymatic activity found chemically in tissues fixed for a short period of time (2 hours) seems to indicate that inactivation progresses with time.

The effect of acetone and alcohol may explain the findings of Bartelmez and Bensley on the negative reaction of Schwann cells in fixed material as compared with positive reactions in unfixed tissue.

All other fixatives studied inactivate acid phosphatase to a greater extent both chemically and cytochemically. When interpreting histochemical results obtained with the acid phosphatase technic, one should not forget that glycerophosphate, as compared with phenylphosphate, is poorly attacked by acid phosphatase.

Effect of the histological procedure. Although our experiments were conducted in a different manner, we agree with Stafford and Atkinson on the decrease in enzymatic activity of blocks subjected to conditions similar to those of the histological procedure. This great lowering of activity can be demonstrated also cytochemically.

On the basis of both chemical and cytochemical results we disagree with Gomori's statement that fixation in acetone protects the enzyme from the inactivating effect of alcohol. Our results indicate that alcohol treatment after acetone fixation causes considerable inactivation of the enzyme. This suggests that alcohol should be eliminated from the schedule altogether. The experiments with homogenates show that embedding will inactivate a large portion of the enzyme. We are faced with several inactivating factors that together can account for a great part of the difficulties encountered in the method for acid phosphatase.

Extraction of enzyme and of lead phosphate. We were able to confirm chemically and histochemically the findings of Bartelmez and Bensley on the extraction of acid phosphatase from fixed tissue. Results were conclusive only after 5 hours of extraction. The fact that only 5% of the enzyme could be extracted by water leads us to think that extraction is not a very important factor in the histo-

chemical method. Under the conditions of our work we could not verify the removal of lead phosphate by water from sections. A slight decrease in color intensity was seen after 10 minutes in absolute alcohol plus 110 minutes in xylene or after 120 minutes in absolute alcohol, in partial agreement with the observation of Moog (1943). Xylene, however, may attack lead sulfide, and to prevent this, sections should be cleared in gasoline or tetrachloroethylene and mounted in Clarite dissolved in the same solvents (Gomori, 1945).

Effect of temperature on enzyme reaction. Our chemical results are in agreement with the histochemical ones, pointing to a certain thermostability of the enzyme. The optimal temperature of enzymatic activity seems to lie between 37 and 61°C. It is possible that carrying out the cytochemical method at a temperature closer to the optimum would contribute to better results. We did not study chemically the effect of temperature on enzymatic activity of fixed tissue, but the agreement between the chemical and the visible histochemical results was good. The fact that at 6°C. the histochemical reaction is decreased in intensity and that saponin does not affect the reaction speaks against a process of mere adsorption.

Apart from the difference in material studied, it is possible that this relative thermostability of the enzyme and its resistance to certain agents, together with adsorption of lead, may explain Lassek's findings (1947). When a process of adsorption is suspected, control sections incubated in the presence of fluoride or without glycerophosphate should be used, and only the difference between the control and regular sections should be considered to be due to enzymatic activity.

Effect of sodium fluoride and other chemical agents. In agreement with Wolf *et al.* (1943), we have shown cytochemically a pronounced inhibition of the reaction by as little as $M/17.000$ of sodium fluoride. In a formol solution, used as a fixative, sodium fluoride did not inhibit the cytochemical reaction. This can be explained by the reversibility of fluoride inhibition (Belfanti *et al.*, 1935).

We could not confirm the results of Moog (1943, 1944) in respect to the histochemical inactivation of enzymatic activity by ferricyanide or the enhancement of the reaction by ascorbic acid and manganous sulfate. Lassek (1947) claims to have obtained cytochemical reactions after the treatment of sections with hydrochloric and nitric acids, ammonia, hydrogen peroxide, phosphotungstic acid, mercuric chloride and picric acid. The effect of these substances on acid phosphatase activity should be checked by test tube experiments, using nervous tissue as the source of the enzyme. In our experi-

ments, ammonia treatment of sections completely inactivated the enzyme histochemically, in agreement with the data of Bamann and Diederichs (1934).

Recently, Doyle (1948) studied the effect of ethyl and methyl alcohol and acetone fixation, temperature of fixation, paraffin embedding and freezing-drying on acid phosphatase in the appendiceal lymph nodes of the rabbit. Our results, although in different material, agree with his.

CONCLUDING REMARKS

The histochemical technic for acid phosphatase, in its present form, is far from being an optimal one. The use of glycerophosphate as a substrate diminishes its sensitivity. The great inactivation produced by fixation, alcohols and embedding, are factors contributing to poor results. These factors may explain, at least in part, the inconsistency of its results.

In general, our results seem to point to the specificity of the method. Comparison with results of other workers is difficult in view of the difference in tissues studied. The possibility of the extraction of the enzyme, although very slight under our experimental conditions, may deprive the method of its strict localizing value, as asserted by Bartelmez and Bensley.

Further study is desirable on this subject with the aim of devising a modification of the procedure which would assure good fixation and preservation of the enzyme.

In the present state of our knowledge it seems that the main points to be observed for optimal results are fixation in chilled acetone, dehydration in the ice box and exposure to the temperature of the paraffin oven for the shortest possible length of time.

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INITIAL AND PERSISTING STAINING POWER OF SOLUTIONS OF IRON-HEMATOXYLIN LAKE

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ABSTRACT.—A study was made of factors affecting the initial staining power and the stability of iron-hematoxylin lake solutions. The findings were applied to the preparation of a superior hematoxylin staining solution. This is made up as follows: in 50 ml. water dissolve, in order, 1.0 g. ferric ammonium sulfate [$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$], 0.8 ml. sulfuric acid, 50 ml. 95% ethyl alcohol, 0.5 g. hematoxylin. Filter the solution to remove the insoluble, white crust of the ferric ammonium sulfate. The solution stains well ten minutes after it has been made. Peak performance is attained within 5 hours, and is maintained for 4 to 8 weeks. Staining time is 3 to 30 minutes. Excess stain can be rinsed off the slide and section by immersion in water, after which destaining, if necessary, can be accomplished with a solution of 50 ml. water, 50 ml. 95% ethyl alcohol, 0.18 ml. sulfuric acid. The slides may or may not be placed next in a neutralizing solution of 50 ml. water, 50 ml. 95% ethyl alcohol, 0.5 g. sodium bicarbonate. They may then be passed through 50 ml. water, 50 ml. 95% ethyl alcohol on the way to alcoholic counterstaining solutions, or through water leading to aqueous counterstains.

The nuclear stain produced is black, intense and very sharp and has proved to be consistently excellent on a variety of animal and human tissues following a number of different fixatives.

The abundance of nuclear hematoxylin staining formulae on record does not, unfortunately, deny the proposition that there is yet much room for improvement. Many of the methods do have their points of excellence; but a happy combination of these points is not available. Cole (1943) recently presented a digest of methods employed in preparing hematoxylin solutions and prescriptions for their most profitable use as stains, along with a comprehensive bibliography on the subject.

The present work had as its goal the development of a hematoxylin solution that would be easy to prepare; stain nuclei rapidly, intensely and selectively under a variety of conditions; and be de-

pendably consistent in performance over a period of several weeks. Previous experience indicated that the most promise dwelled in the iron-hematoxylin lake.

In an iron-hematoxylin lake solution with laudable initial staining ability, the retention of this property obviously will depend upon the stability of the solution. The task at hand was thus a dual one involving, first, a study of factors determining the initial staining attributes of an iron-hematoxylin lake solution, and second, a study of factors influencing retention of staining power, which is tantamount to a consideration of factors affecting stability. These studies have, in fact, provided the data for formulating an improved stain. Beyond this, they have shed some light upon the chemistry of hematoxylin staining, which subject, however, is outside the scope of this report.

The procedure employed consisted of determining what the factors are that influence the initial and persisting staining power of an iron-hematoxylin lake solution, and then systematically preparing and testing solutions to find the optimum adjustment of each. The hematoxylin used (National Aniline Division, Allied Chemical and Dye Corporation) was Commission certified. Three different lots were employed. Though the color of the crystals varied from a light chocolate brown to a pale straw yellow, all lots yielded similar results. When a high initial content of hematein was desired in the solution, the dissolved hematoxylin was oxidized with mercuric oxide.

Initial staining ability of an experimental solution was determined by staining tests on a wide variety of human material. Adjacent tissue sections were employed whenever direct comparison of staining abilities was essential. Stability of the staining solution was judged subjectively by the amount of film deposited on the surface of the liquid, by the coating on the glass container, by the precipitate formed, by color changes determined with test strips of bibulous paper, and by staining performance. Occasionally, precipitates were dried and weighed. Determinations of pH were made with a Coleman model G pH meter.

FACTORS AFFECTING INITIAL STAINING POWER AND STABILITY

The concentrations of ferric ion and of hematoxylin or hematein. In aqueous solutions between pH 1.90 and 0.80, it was demonstrated that increasing the ferric ion concentration or that of the hematoxylin or hematein, or both, decreased the stability of the solution. Decrease in persisting staining power was manifested both by fainter staining of nuclei and by darker staining of background with passage of time. On the other hand, the concentrations had to be sufficient to produce

a stain of adequate intensity. Whether the solution was prepared to contain initially only hematoxylin or a high proportion of hematein made extremely little difference in initial staining performance.

The pH of the solution. Increasing the hydrogen ion concentration, with other conditions maintained constant, increased the stability and the persisting staining power of solutions. Three series prepared with different acids—orthophosphoric, sulfuric and hydrochloric—yielded similar evidence. The sulfuric acid series may be used as illustration. The test solutions were made up with 50 ml. water, 0.25 g. hematoxylin, 0.5 g. ferric ammonium sulfate [$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] and amounts of sulfuric acid (sp. gr. about 1.84, not less than 94% H_2SO_4) varying from zero to 0.5 ml. In solutions of pH greater than 1.50, heavy precipitates formed almost immediately. The precipitate was heavier and formed more rapidly at the higher pH values. Solutions of lower pH contained 0.10, 0.20, 0.25, 0.30, 0.40 and 0.50 ml. sulfuric acid providing pH values of 1.33, 1.08, 1.01, 0.91, 0.83 and 0.71 respectively. The solution of pH 1.33 showed moderate film and precipitate almost immediately which became heavy in 4 hours. The solution stained well at first but was much less efficient—fainter and less selective—at 1 hour and poor at 4 hours. The solution at pH 0.71 showed no evidence of change at first. At 3 days there was a moderate film which was heavy at 6 days and accompanied by a slight coating. Even at 24 days there was no precipitate. Staining was good for three days, after which it slowly declined, but even at 24 days was not as poor as the performance of the pH 1.33 solution at 4 hours. The intermediate solutions yielded stability and staining data that fell in line between these two at the extremes.

It was apparent that the lowest pH values determined the most selective staining, other conditions being comparable. The most tenable generalized statement covering the mechanics of ordinary staining is that cationic dyes stain anionic tissue elements, and anionic dyes color cationic tissue elements, be the process one of adsorption or of chemical union. For a brief discussion of the theory of staining the reader is referred to Conn (1946). The chemical union view was supported by Craig and Wilson (1937). In connection with the action of iron in staining with hematoxylin these authors suggested that a complex is formed whereby iron binds hematoxylin to protein. Regardless of just how the attachment occurs it was abundantly evident in the present work that as pH was lowered and anionic tissue elements were progressively eliminated, the cationic iron-hematoxylin lake progressively became a more selective nuclear

stain, and, eventually, a weaker one. Whatever the substances the lake stains in the fixed nucleus—nucleoproteins or hydrolytic products—some, at least, were still anionic at pH 0.60, the lowest tested.

The pH of the solution played an important role in the intensity of the nuclear stain in initial trials. At a relatively high pH, where the solution deteriorated almost at once, staining was weak. Relatively low pH values resulted in weak nuclear staining, too: for one thing, anionic nuclear elements might have been attenuated to an extreme, and for another, the concentration of the iron-hematoxylin lake probably was low in this circumstance.

The nature of the anions in the solution. Anions in the solution originate from the acid and from the ferric salt. Since the foregoing considerations indicate the desirability of a low pH, a strong or moderately strong acid and the ferric salt of such an acid are necessary. Nitrate was eliminated on the basis that nitric acid is a capable oxidizing agent even in dilute solutions, and it was thought that the life of the hematoxylin solution might be unnecessarily abbreviated by it. Phosphate proved impractical because, while ferric phosphate was soluble at the pH of the staining solution, it precipitated on the section when the latter was rinsed in distilled water. Oxalate failed: solutions were clear, very pale yellow and did not stain. It is probable that the oxalate, by reduction, freed the solution of the chromophore and of ferric ion. Chloride and sulfate remained as the likeliest possibilities, and of the two, sulfate turned out to be the better. Hydrochloric acid or ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) or both were used to introduce chloride ion into the solution, and sulfuric acid or ferric ammonium sulfate [$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$] or both were employed for sulfate. Sulfuric acid and ferric ammonium sulfate were more convenient to handle in preparing the solutions than were hydrochloric acid and ferric chloride. Sulfuric acid was favored also for its non-volatility. There was some suggestion that chloride ion added to the instability of the solution, but it was not deemed pertinent to the study at hand to confirm this fully at the moment.

The nature of the solvent. Replacement of water as the solvent by a 1:1 mixture, by volume, of water and 95% ethyl alcohol, markedly increased stability of solutions. Initial staining was about the same in the two solvents. The increase in stability conferred by this solvent did not alter the character of the influence exerted by the other factors affecting stability which have been discussed above. Test solutions were made up to contain 25 ml. water and 25 ml. 95% ethyl alcohol. Sulfate was established as the anion of choice. Quantities of sulfuric acid between zero and one milliliter were em-

ployed in determining effect of hydrogen ion concentration. In the appraisal of the effects of ferric ion and of hematoxylin concentrations, 0.25 g. hematoxylin (making the solution approximately 0.014 molar), 0.40 g. hematoxylin (making the solution approximately 0.022 molar), and double these amounts were used in the several possible combinations with 0.35 g. ferric ammonium sulfate (making the solution approximately 0.015 molar with respect to ferric ion), 0.50 g. of the latter (making the solution approximately 0.021 molar with respect to ferric ion), and double these concentrations.

PREPARATION AND USE OF THE STAIN

The foregoing findings cleared the path to the prime objective of this study, namely, the preparation of a better hematoxylin staining solution employing the iron-hematoxylin lake. The solution should be made with a solvent of 1:1 by volume water, 95% ethyl alcohol; sulfate is the preferable anion. The optimum, practical concentrations of hydrogen ion, ferric ion and hematoxylin were still to be determined.

Information gleaned partly from results herein reported and partly from ancillary experiments suggested that ferric ion and hematoxylin react to form a definite compound, iron-hematoxylin lake, and hydrogen ion. The lake, in solution, is the stain. As would be expected, low hydrogen ion, high ferric ion, and high hematoxylin concentrations favor formation of the lake. If there were no other pertinent factors, it would be necessary merely to set conditions that would result in a satisfactory yield of lake. But complications are rife. Solubility of the lake may vary with conditions in the solution. Side reactions are manifest which result in alteration of the lake or in formation of products which detract from the staining performance of the solution. Furthermore, as has been discussed, careful adjustment of the hydrogen ion concentration is essential in order that the stain be both selective and intense. As a result, some compromises were necessary to get sufficient concentration of lake with sufficient freedom from undesirable side reactions with proper hydrogen ion concentration.

A solution made up as follows is the most favorable for the establishment and retention of effective staining power once an adequate supply of iron-hematoxylin has formed:

- 50 ml. distilled water
- 0.8 ml. sulfuric acid (sp.gr. about 1.84, at least 94% H_2SO_4)
- 1 g. ferric ammonium sulfate [$\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$]
- 50 ml. 95% ethyl alcohol
- 0.5 g. hematoxylin (Nat. Aniline Div.; Commission Certified)

The order given is the most satisfactory one to observe in adding components. The ferric ammonium sulfate dissolves at room temperature in a few minutes with stirring, so that no time is saved by warming the solution since then it must be cooled and restored to volume. The ferric ammonium sulfate should be completely dissolved (except for the white crust on the crystals, which, if present, remains as a residue) before addition of ethyl alcohol, since it dissolves but very slowly afterwards. The sulfuric acid must precede the hematoxylin in order that the hydrogen ion concentration be optimum before the reactants are mixed. The sulfuric acid is handled easily, safely and accurately by pouring the approximate amount required from a small storage bottle into a 5 or 10 ml. beaker, from which the acid can be drawn into a glass syringe and the required volume delivered. The ethyl alcohol should be added before the hematoxylin. The latter then dissolves in a few seconds with stirring. The solution is filtered. The pH at completion is 1.04 ± 0.03 .

It is not known when the reaction yielding iron-hematoxylin in this solution reaches equilibrium; but it is apparent that sufficient of the compound is formed within ten minutes to stain very well. In the interval between 10 minutes and 5 hours after preparation of the solution, its staining performance, though submaximal, is nevertheless very commendable and quite satisfactory in most instances. In no case will it fail to give a usable stain, but with some tissues the nuclear stain may be slightly lighter than that yielded by a solution over 5 hours old. After 5 hours, the staining performance is at a peak, and this peak level is maintained for 4 to 8 weeks. During this interval, a slowly progressive change in the solution is mirrored by an increasing intensity of background staining in the tissue, i.e., diffuse staining of elements other than nuclei. At first, the background is unstained. With passage of time it becomes gray-tinged, which coloration gradually darkens. Finally, and presaging the close of the period of usefulness of the stain, the background becomes a dirty yellow.

Staining time varies from 3 to 30 minutes. Longer periods than this, even days, do not give adverse results, but of course they are unnecessary. Following staining, excess of the dye solution can be rinsed from the section and slide by immersing them in two changes of either distilled water or tap water. The gray color in the background can always be removed completely by destaining in the following solution: 50 ml. distilled water, 50 ml. 95% ethyl alcohol, 0.18 ml. sulfuric acid. The pH of this decolorizing solution is 1.67 ± 0.03 .

When the staining solution has exceeded its useful span, the nuclei no longer stain intensely enough, and the gray-yellow background is difficult to decolorize. Following destaining, it is sometimes advantageous to place the section in a neutralizing solution of: 50 ml. distilled water, 50 ml. 95% ethyl alcohol, 0.5 g. sodium bicarbonate. Next, the section may be placed in a solution of 50 ml. distilled water, 50 ml. 95% ethyl alcohol on the way to alcoholic counterstaining solutions, or in distilled water on the way to aqueous counterstains.

The volume of the staining solution should not be allowed to become reduced by evaporation by more than 40% of its original volume. Losses up to 40% may be restored by addition of an 80% ethyl alcohol solution. Losses from the acid destaining and alkaline neutralizing solutions may be compensated similarly.

The nuclear stain produced by the method described is black, intense, and very sharp. It has proved to be consistently excellent on a variety of tissues fixed with 10% formalin; Bouin's; Zenker's; and a modification of the latter containing acetic acid, formalin, ethyl alcohol and isopropyl alcohol. The staining solution has been compared with numerous other hematoxylin solutions as to ease of preparation, convenience of use, length of life, and staining results on similar sections and in combination with various counterstains. It has been found superior to all in the writer's judgment. It may be noted that Weigert's iron hematoxylin gives somewhat comparable results over a much briefer span. On the basis of the present work, the limitations in the value of Weigert's stain can be ascribed precisely to certain faults in the composition of the solution.

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OBSERVATIONS ON THE STAINING OF CORYNEBACTERIUM DIPHTHERIAE

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ABSTRACT.—Albert's method of staining diphtheria cultures consists of staining a fixed smear for one minute (some laboratories stain for five minutes) with a solution containing toluidine blue and malachite (or methyl) green, washing with water, and applying Albert's iodine for one minute. This procedure is discussed and criticized, and in addition the mechanism of the stain is elucidated. Also, the procedure which involves staining a fixed smear for one minute with Loeffler's alkaline methylene blue solution is discussed and criticized.

To overcome the objections to the above staining methods, a different method is proposed. This consists of staining a fixed smear with an acid solution of toluidine blue, washing with water, applying Albert's iodine for one minute, washing with water, and finally applying a safranin solution for 15–20 seconds. The theoretical basis for this method is presented.

Mueller and Miller (1946) pointed out that the decreasing prevalence of diphtheria in many localities has led to the development of a generation of bacteriologists with but little experience in the identification of the causative organism. The writer, prior to taking his present position in a public health laboratory, had but superficial experience with the diphtheria organism, and so was definitely a member of this "diphtheritically lost generation". After a year, however, during which a few sporadic cases of diphtheria have occurred in the community, with the attending deluge of throat cultures, the popular Albert's (1920, 1921) and the classical methylene blue methods of staining smears from Loeffler cultures have come to be viewed with certain dissatisfaction. Consequently, a method was worked out for staining diphtheria cultures which eliminated the objectionable characteristics of the above methods.

The objectionable features of Albert's stain and methylene blue have been stated to some extent by other workers, who usually prefer one or the other method, but they may be stated briefly according to the writer's experience as follows:

Albert's Stain. The staining solution is a mixture of toluidine blue

and malachite (or methyl) green in an aqueous solution containing ethyl alcohol and acetic acid. This solution when first prepared yields a rather considerable precipitate, the formation of which not only decreases the staining capacity of the solution, but also, considering the variation in dye content of different preparations of the stain powder, will result in unavoidable variation in the staining capacity of different solutions.

The stain is essentially a granule stain, and as such, stains only the granules with any reliable uniformity. Much too often the diphtheria cell body is stained too faintly to detect the protoplasmic striations (barring), and in fact at times even the outline of the diphtheria organism is indistinct. In Loeffler cultures, therefore, especially to the beginner, confusion can and does arise as to whether the indistinctly stained, frequently occurring, granulated cocci, appropriately spaced, might not be diphtheria bacilli.

Other bacillary forms which produce granules, if the bacteriologist is not conversant with the finer inner structure of the diphtheria cell, and with the arrangement of the organisms in the microscopic field, can very easily be confused with diphtheria bacilli.

Methylene Blue. This stain can be prepared with uniform staining capacity, and it eliminates some of the objections to Albert's stain. Thus, protoplasmic striations (barring) are shown clearly, and contaminating organisms are stained distinctly enough to prevent confusion with diphtheria organisms. The stain, however, demonstrates only well developed granules, and in the case of an organism which does not show protoplasmic striations, and in which the granules have not yet developed to the stage where they are detectable with methylene blue, the organisms will stain solidly and have none of the inner distinctive features of the diphtheria organisms. It might be added in this connection that the granules of the diphtheria organism are composed of nucleic acid and are slowly formed by metabolic cell processes; the initial tiny granules are easily overlooked with methylene blue, but can be shown by a staining method presented in this paper.

From the above observations, it appeared that a stain to be adequate for use on diphtheria cultures, particularly for bacteriologists with little experience, must demonstrate uniformly: (1) the complete morphological outline of the cell body, (2) the presence or absence of protoplasmic striations, and the presence or absence of granules, no matter how small, and (3) stain contaminating organisms clearly to avoid confusion with the diphtheria organism.

EXPERIMENTAL

Inasmuch as Albert's stain will at times demonstrate both protoplasmic striation and granules, it was decided to investigate the mechanism of this stain, with the object of modifying it to fulfill the requirements listed above. In this study, pure cultures kindly supplied by Dr. Martin Frobisher, Jr., together with a local strain, were used.

Two staining solutions were prepared. One contained toluidine blue in the same proportions with acetic acid, alcohol and water as is found in Albert's stain, and the other contained malachite green, also in the same proportions as in Albert's stain. Fixed smears of the diphtheria organism stained with the toluidine blue solution showed that both protoplasmic striations and granules were stained clearly, with the granules being a considerably darker blue than the rest of the cell. However, with the malachite green solution, the cells were stained uniformly and faintly green and showed neither striation nor granulation. When Albert's iodine was then applied to the stained smears, in the case of the toluidine blue stain, no effect was observed on the striations, but the granules appeared to be somewhat darker. The iodine had no effect on the smears stained with malachite green alone.

Another series of fixed smears was then prepared, stained first with toluidine blue, washed and then stained with malachite green. With this treatment, the malachite green removed the toluidine blue from the protoplasm of the cells, which became light green, with only faint differentiation of the protoplasmic striations. The granules, however, retained the toluidine blue. Application of Albert's iodine then changed the granules from blue to black, with additional decrease in the intensity of the malachite green stain in the protoplasm.

The above simple experiments indicate that in the regular Albert's stain, the malachite green is held by the bacterial protoplasm in preference to toluidine blue, whereas the toluidine blue preferentially stains the granules. The function of Albert's iodine appears to be merely one of increasing the intensity of the granule stain, possibly by formation of a black, insoluble iodine-toluidine-blue oxidation product. This supposition is borne out by the fact that in the test tube, a mixture of Albert's iodine and toluidine blue quickly yields a black precipitate. As indicated above, however, the use of iodine as the final reagent is not desirable since it results in a decrease in the staining intensity of the malachite green. This can probably be explained on the basis of acid production in the iodine solution, which would tend to wash out the stain.

In view of these observations, it was felt that replacement of malachite green with a stain possessing greater affinity for the bacterial protoplasm would result in better and more consistent differentiation of the protoplasmic striations. The ordinary safranin used in the Gram stain was chosen. Slides prepared and stained first with the toluidine blue solution, washed and then stained for 30 seconds with safranin, showed that this dye also replaced the toluidine blue in the protoplasm, but left the latter dye in the granules. Further, the protoplasmic striations were very much more distinctly stained than with Albert's method. It was observed, however, that there were somewhat fewer granules in some cells, and it was apparent that the safranin removed the toluidine blue from some of the smaller granules, leaving them, not blue, but instead a darker red than the rest of the cellular protoplasm. To eliminate this replacement of toluidine blue, advantage was taken of the action of iodine on toluidine blue. By applying Albert's iodine for one minute to the preparation already stained with toluidine blue, washing and then staining with safranin, the toluidine blue was "fixed" in even the smaller granules, which then appeared black and even more distinct in the red cell body. It might be added at this point, that Neisser's stain, consisting of methylene blue solution followed by Bismarck brown or safranin, would probably also suffer from replacement of the methylene blue by any possible overstaining with the other dyes.

The staining method developed from the above experiments consists essentially at this stage of staining with the original Albert (1920) formula, washing, applying Albert's iodine, washing, and staining then with safranin. However, Morton and Francisco (1942) showed that increased acidity in granule stains for the diphtheria bacillus resulted in a better differentiation of the cellular structures. To test the effect of increased acidity on the present method, preliminary experiments were made in which acetic acid concentration of the toluidine blue solution was varied from the original concentration of 1.0 ml. per 100 ml. of staining solution, to 2.5, 5.0 and 10.0 ml. Fixed smears stained with these solutions, followed in the usual manner by iodine and safranin, showed no better differentiation with 2.5 ml. of acetic acid as compared to 1.0. With 5.0 ml. however, there was sharper differentiation of some structures, whereas 10 ml. brought no additional improvement.

The experiment was then repeated, using only 1.0 and 5.0 ml. of the acetic acid. Nine different strains of the diphtheria organism (including *mitis*, *gravis*, *intermedius* and *minimus* types) were used. In a majority of cases the 5.0 ml. concentration of acetic acid resulted in

a much sharper differentiation of the protoplasmic striations, a phenomenon which indicates that acetic acid renders the weakly stainable bands in the diphtheria cell even less stainable by the basic dye safranin. Also the increased acetic acid concentration resulted in some cases in a decrease in the staining intensity of the safranin, thus permitting the black granules to stand out more clearly. The granules themselves, however, appeared to be unaltered by the various concentrations of acetic acid used.

The results of all the above experiments thus led to the following formula and technic, which fulfills to the best advantage the three requirements listed in the introduction.

I. Toluidine blue, certified (52% dye content)...	0.15 gram
Glacial acetic acid.....	5.0 ml.
Ethyl alcohol, 95%.....	2.0 ml.
Distilled water.....	100 ml.

The stain is ready for use immediately following complete solution. Filtering is not necessary.

II. Albert's iodine

III. Safranin (As prepared for the Gram stain)

Method. Stain smear with I for 1 minute. Wash with water. Apply II for 1 minute. Wash with water. Stain with III for 15-20 seconds. Wash, dry and examine. Protoplasmic striations when present are always distinctly shown, and usually stain red with a light pink to almost colorless band of material between. Occasionally they may stain a reddish brown. Granules are very distinct and stain black, and in cells with no striation, the protoplasm stains a light pink.

The staining method presented above, though original with the writer, cannot, of course be considered a completely new method. Subsequent review of the literature has yielded the information that all reagents used have been used before in different combinations and modifications by earlier workers (Albert, 1920, 1921; Laybourne, 1924; Weiss, 1929; Kemp, 1931), though in no case was the theory behind these modifications presented. Therefore, so far as is known, the proportions and order of the staining reagents presented in this communication, have not been presented before. It is felt that the present method is preferable to either Albert's method or methylene blue, particularly for bacteriologists with limited experience with the diphtheria bacillus, and also for college and university classes in bacteriology for demonstrating with one method the finer structure of the diphtheria cell.

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✓ SOME MODIFICATIONS OF WARMKE'S PERMANENT SECTION-SMEAR METHOD FOR PLANT CHROMOSOMES¹

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ABSTRACT.—A modified schedule for making permanent section-smears is given. This incorporates numerous minor changes such as precooling the 1"-1½" roots for 3 hours at 0°C., fixing in cold Randolph's fixative, using a partial vacuum for ten minutes, thorough washing after fixation, chloroform infiltration, 15-30 minutes in leucobasic fuchsin followed by washing in water for one to four hours, double staining with crystal violet to obtain intense staining of special value in making photomicrographs, and a different method for smearing soft root tips with small chromosomes. Permanent sections stained only with crystal violet, without hydrolysis, can be smeared; this improves the accuracy of counts on difficult material and is useful when rapid routine methods are employed in the study of large numbers of plants.

The section-smear method of Warmke (1941 and 1946) was used on fixed anthers and root tips of *Lobelia Cardinalis* and satisfactory preparations were obtained. The root-tip tissues of *Agropyron pectiniforme* were found to be much tougher and harder; experiments were undertaken to try to find a method for softening them. The experiments were later extended to improve the staining, smearing, and a simplification of the schedule.

Various diploid and tetraploid species of *Lobelia*, diploid, triploid, tetraploid, and hexaploid plants of *Agropyron*, as well as miscellaneous species including *Asimina triloba*, *Crepis capillaris*, and *Begonia Evansiana*, have been used as experimental material.

Numerous minor changes and improvements were made and wherever possible, less expensive materials were used, such as Randolph's fixative. The root tips precooled at 0°C. were kept living and large-diameter roots such as those of *Agropyron* were cut long enough to avoid adverse physiological effects; roots should be cut at least 1" to 1½" long. A more marked shortening of the chromosomes was obtained by precooling for 3 hours rather than for 1½ hours. Thorough washing after fixation improved the softening

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of the tissues. The chromosomes were more intensely stained when kept in the leucobasic fuchsin for 15 to 30 minutes rather than for 10 minutes. The sulphurous acid wash was omitted. Washing for 1 to 4 hours in water, following the leucobasic fuchsin, softened the tissues.

Three staining procedures were employed: the Feulgen reaction, the crystal violet method, and a combined staining schedule, Feulgen and crystal violet. Chromosomes stained with the Feulgen reaction appeared brilliant light purple (cyclamen purple); with crystal violet, they were blue-violet to reddish-purple; and with the Feulgen and crystal violet, the chromosomes were light bluish-purple to deep reddish- or violet-purple. The combined method increased the intensity of staining and the contrast of the chromosomes; a wide range of contrasts was obtained by adjusting the amount of destaining. The chromosomes of some genera absorbed and held the crystal violet more strongly than others. For the preparation of especially good smears or for making photomicrographs, the double stain, Feulgen and crystal violet, was best; the method is described in Schedule A.

For purposes of photomicrography, it is necessary to have high contrast between the stained chromosomes and the clear background. There must also be good differentiation of chromosome structure. In chromosomes stained with the Feulgen reaction, the details of structure are excellent but the intensity of staining is less than with the combined staining schedule; with the latter, every slide is well stained. Chromosomes are usually densely stained with crystal violet but may lack differentiation of structure. The Feulgen reaction chemically stains the desoxyribose-nucleic acid throughout the whole structure of the chromosome and the crystal violet is absorbed. The Feulgen reaction increases differentiation because the internal structure of the chromosome is much better stained than with crystal violet.

If the Feulgen reaction is desired, steps 8 to 10 are omitted, and the ascending series of alcohols are added in Schedule A. In this laboratory, permanent sections stained with crystal violet are used for making rapid, routine counts on large numbers of plants. It has been found that metaphases in these slides can be smeared, even though there has been no hydrolysis; this has increased the accuracy of making chromosome number determinations on difficult material. The procedure is described in Schedule B.

La Cour (1947) has given a general account of recent developments in smearing. Lee (1937) is a valuable source reference for cytological procedures.

SCHEDULE A

This schedule is recommended when especially good permanent preparations are desired showing the range of chromosome size and morphology, or when it is desired to make photomicrographs. The procedure is:

(1) Place 1" to 1½" mitotically-active root tips in vials half-full of water in the 0°C. refrigerator for 3 hours. Avoid direct contact of the vials with the cold metal lining of the refrigerator.

(2) Place vials in dish of ice-cold water. Transfer to room temperature and cut root tips ⅛" to ¼" long. Fix in vials of cold Randolph's fixative, using a partial vacuum for the first ten minutes. Keep the vials in the 0°C. refrigerator for two hours and then at room temperature for 22 hours. Wash in water for 2 to 4 hours.

(3) Dehydrate in 50% alcohol for ½ to 1 hour, place in 70% for 1 hour, or store until needed. Complete dehydration, and embed by the chloroform method, but avoid excessive heat. Use rubber-parowax embedding and section at 15μ for small and medium-sized chromosomes.

(4) Deparaffinate in xylene for one minute and dioxane for one minute. Pass the slides quickly through the descending series of alcohols to water. Soak in water for one to four hours.

(5) Normal hydrochloric acid at 60°C. for 45 minutes.

(6) Leucobasic fuchsin for 15 minutes. *Agropyron* root-tip metaphases are stained more deeply after 30 minutes but there is an increase in the hardness of the tissues. A few crystals of potassium metabisulfite may be added to the stain.

(7) Wash in water for one to four hours.

(8) Stain in 1% boiled and filtered aqueous crystal violet for 15 minutes. Rinse quickly in water.

(9) Apply 1% iodine and 1% potassium iodide in 80% alcohol for 30 seconds.

(10) Destain in two changes of 95% alcohol for 10 seconds each.

(11) Destain further in dioxane. Clear in two more changes of dioxane for a total of 5 minutes.

(12) Using a pipette-bottle, rinse sections with very thin dioxane-balsam.

(13) Mount in thin dioxane-balsam. Dry slide at room temperature until dry enough for best smearing, about three to ten days.

Root tips of *Lobelia* are much softer than those of *Agropyron*, and different smearing methods must be used for these two genera. For *Lobelia*, tap lightly with a lightweight scalpel or probe, almost directly over the metaphase plate. It is not desirable to free the

cell from the rest of the section. Smear and spread the chromosomes to obtain optimum separation in a thin, flat plate on the same horizontal plane; see Fig. 1B. If too much pressure is used, chromosome structure will be altered.

For root-tip tissues of *Agropyron*, the selected cell must first be freed from the rest of the section by tapping as described by Warmke (1941). Then smear and tap to separate and spread the chromosomes; finally, use sufficient pressure to fix the chromosomes in the same horizontal plane; see Fig. 1A.

There are numerous advantages with this method, when skill has been acquired through practice: the slides are permanent; chromo-



FIG. 1.—Photomicrographs of permanent section-smears of mitotic metaphases in root tips stained with Feulgen and crystal violet, using a combination of Wratten filters No. 60 (green) and No. 22 (deep orange). (A) *Agropyron pectiniforme* R. & S., $2n=14$. (B) *Lobelia Cardinalis* L., inter-varietal hybrid, $2n=16$, diploid complement with two small centric chromosomes. Note the two SAT-chromosomes at 1 and 9 o'clock in A, and at 2 and 4 o'clock in B.

some structure can be observed before and after smearing; staining and fixation are excellent; and large numbers of metaphases are available in each slide [for example in a slide of *Crepis capillaris* ($2n=6$), three satisfactory metaphase smears were quickly obtained in one section].

SCHEDULE B

Many plants are examined cytologically in this laboratory each year and permanent sections stained with crystal violet have proved to be the most satisfactory for rapid routine determinations on root tips. Some of the materials are much more difficult than others. For example, collections of Arctic grasses were examined recently and

a range of chromosome number of $2n=14$ to $2n=140$ was determined. It was found that these permanently-stained crystal violet sections could be smeared fairly well, even without hydrolysis. Such section-smears were particularly valuable, first, in a few slides in which the chromosomes tended to stick or clump together; and, second, in species with a very high chromosome number.

The same procedure is followed as for Schedule A, except that steps (5) to (7) are omitted; and the following steps are added between steps (10) and (11): (i) Destain further in absolute alcohol for 15 seconds and (ii) clear in clove oil for 5 seconds or until sufficiently destained. Step (12) may be omitted, if desired, to shorten the method.

To improve the intensity of staining with crystal violet, leave the slides in each change of 95% alcohol for only 5 seconds each, in absolute alcohol for 5 to 10 seconds, in clove oil for 2 to 5 seconds, and in the first dioxane for 10 seconds. Do not leave for too long a time in the first dioxane since some clove oil is brought over with each slide. Complete destaining and clearing in the 2nd and 3rd dioxane (5 minutes), and mount in dioxane-balsam. Soft root tips can be smeared at once; others should be dried for several days before any smearing is attempted. Best smearing results with routine slides were obtained when the balsam had dried for about one week. The smearing is only partly successful with harder root tips, such as *Agropyron*; good smears can be made with softer materials like *Lobelia*.

The stain is much less intense when crystal violet is used without the Feulgen reaction. The procedure of staining is much more rapid than Schedule A and also more rapid than the Feulgen method. Except in very soft root tips, the metaphases cannot be smeared as well as those done by Schedule A. If rapidity and accuracy in determining large numbers of plants are the only requirements, the crystal violet schedule is more practicable as a routine method. Because of less intense staining and less flattened smears except in the softest root tips, crystal violet smears are not as good for photomicrography as Feulgen-crystal-violet smears.

The following materials could be smeared with the crystal violet method: *Asimina triloba* ($2n=18$); *Crepis capillaris* ($2n=6$); *Lobelia Cardinalis* ($2n=14, 15, \text{ and } 16$); *Begonia Evansiana* ($2n=26$); *Agropyron ungavense* ($2n=28$); *Calamagrostis* ($2n=140$); *Poa* spp. ($2n=84, 2n=112$); and *Festuca* sp. ($2n=56$). When the metaphases were smeared, there was a marked improvement in the spreading and separation of the chromosomes. The method was helpful in making

accurate counts on the high polyploid grasses, and in studying chromosome morphology.

The Feulgen, Feulgen-crystal-violet, and crystal-violet staining schedules were used to make permanent section-smears of anthers of *Lobelia Cardinalis*. Staining and smearing results were comparable to those described for root-tip material.

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SUDAN BLACK B—A NEW STAIN FOR CHROMOSOME SMEAR PREPARATIONS¹

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ABSTRACT.—Various combinations of Sudan black B (SBB) in some of the lower fatty acids and related acids have been used to stain the chromosomes of smear preparations of onion root-tips prefixed in 2 parts of absolute ethyl alcohol and one part of glacial acetic acid. Temporary preparations were obtained in which the chromosomes and nuclei stained brown but appeared black, when observed with a suitable green filter. Remarkably sharp chromone-matic staining was achieved.

A mixture consisting of equal parts of 1% SBB in 85% lactic acid (LSBB), full strength propionic acid, 1% SBB in 20% formic acid (FSBB) and water added slowly with stirring gave consistently fast and sharp staining with a minimum of dye precipitation. A mixture of equal parts of 1% SBB in 60% propionic acid (PSBB) with LSBB possessed some merit.

A 0.5% solution of SBB in 50% acetic acid yielded an image that resembled the acetocarmine image but did not appear to be quite as useful as the above mixtures. Various solutions of SBB in citric, tartaric, glyceric and glycolic acids were tested. They did not compare favorably with the solutions of SBB in formic, acetic, propionic and lactic acids.

What appeared to be varying chromophoric states of SBB are reported. The blue state did not stain nuclei and chromosomes. They are stained best by the dark red-brown chromophoric state in the presence of adequate water.

INTRODUCTION

The discovery was made recently that Sudan black B (SBB) when dissolved in concentrated lactic acid would stain onion nuclei and chromosomes brown while the nucleoli did not stain (Cohen, 1948). Up to then, SBB had been used chiefly as a fat stain giving a dark blue or a blue-black coloration. Preliminary experiments in which SBB was dissolved in concentrated acetic acid (ASBB) showed that

¹This work was begun in the summer of 1948 at the Marine Biological Laboratory, Woods Hole, Massachusetts.

this mixture was less useful than the lactic acid mixture of SBB (LSBB). The question then arose whether some other acids related to lactic acid might yield superior staining mixtures. It was believed that such information would help in ascertaining the useful limits of SBB as a chromosome stain. In spite of a tendency towards precipitation from solution, SBB showed decided promise as a sharp nuclear stain when observed with the proper filters. The following series of acids was devised for testing in which due consideration was given to their chemical structure, solubility in water and absence of objectionable odors:

citric	$(\text{COOH})\text{CH}_2\text{C}(\text{OH})(\text{COOH})\text{CH}_2\text{COOH}$,	<i>d</i> or <i>l</i> tartaric	$\text{HOOC}(\text{CHOH})_2\text{COOH}$,
propionic	$\text{CH}_3\text{CH}_2\text{COOH}$,	lactic	$\text{CH}_3\text{CHOHCOOH}$,
glyceric	$\text{CH}_2\text{OHCHOHCOOH}$,	acetic,	CH_3COOH ,
glycolic	CH_2OHCOOH ,	formic	HCOOH .

MATERIAL AND METHODS

The usual availability of the common onion bulb, *Allium Cepa*, familiarity with its nuclear appearance (Cohen, 1937) and a large literature for comparison of results favored its use in these experiments. Corroboratory work has been initiated on bean, corn and trillium root-tips.

Sudan black B, product of the National Aniline Division (Certification number NZb-1), was used. The dye solutions were made by boiling and were filtered when cool. Hence, the given dye concentrations in the following solutions are merely approximations. Filtration did not guarantee continued freedom from dye particles since they would reappear in those solutions of acids with high volatility. Except where otherwise noted, 100 mg. of SBB were dissolved in 10 ml. of the organic acid tested to give an approximate 1% solution. In modifying the reported mixtures for use on other material, decrease of the dye content will tend to minimize precipitation of the dye where extremely rapid staining is of secondary importance.

Root-tips and thin slices of scale meristems were fixed in a mixture of 2 volumes of absolute ethyl alcohol and one volume of glacial acetic acid for one-half hour. The material was then transferred to 70% ethyl alcohol. This fixative seemed to preserve the mitotic figures and the details of chromonematic structure better than the mixture incorporating propionic instead of acetic acid.

Maceration of the fixed material in a mixture of equal parts of concentrated HCl and 95% ethyl alcohol was not used, since it seemed to interfere with the subsequent staining. Otherwise the

commonly known manipulations were utilized in making the temporary preparations.

Earlier observations were made using a Wratten X-1 number 11 green filter in combination with a blue daylight filter. Later it was learned that 4 thicknesses of green gift-wrapping cellophane served just as well. The use of a green filter in visual and photographic work is highly desirable since the chromosomes usually appear black on a green background following most of the stain mixtures tried.

RESULTS

Based upon practical utility, the eight organic acids tested as solvents for Sudan black B could be arranged in two groups. Formic, acetic, propionic and lactic acids possessed certain properties that could be adapted for useful chromosome staining in spite of some undesirable features due to the inherent properties of SBB. The remaining acids were not suitable for varying reasons as summarized in Table 1. In all of the acids tested, an adequate concentration of water had to be present in order for suitable staining to occur.

LACTIC ACID

Lactic acid was the first reagent tested with SBB when chromosomal staining was obtained (Cohen, 1948). Lactic acid 85% was used to make up approximately a 1% solution of SBB (LSBB). When LSBB was used with *N* butyl alcohol in a 9 to 1 ratio as a fixative and stain on fresh root-tips, nuclei and chromosomes stained red-brown, while the nucleoli and cytoplasm did not stain. However, alteration of chromosome structure in which despiralization was most pronounced limited the use of this mixture alone as a fixative. The *N* butyl alcohol was incorporated into the mixture to prevent stain precipitation.

When prefixed root-tips were stained in a mixture of LSBB and *N* butyl alcohol, slightly undersaturated with water in the ratio of 2 to 1, remarkably sharp staining of the chromosomes was obtained as observed through the filter combination. Chromonematic structure and details of coiling during the various stages of mitosis were brought out with diagrammatic clarity.²

Preparations incorporating high concentrations of lactic acid are best observed as soon as made. In about a week or so, the stain tends to disappear. (See also Zirkle, 1947). Lactic acid has the advantage of delaying the drying out of temporary preparations. It

²Demonstrations were given at the General Scientific Meeting of the Marine Biological Laboratory, Woods Hole, Massachusetts, on August 24, 1948.

has been used in other technics as a dye solvent and mounting medium [Gueguen (1906), Krause (1926), La Cour (1947), Manneval (1936)]. Lactic acid, when mixed with formic or propionic acid or both, seemed to lower their rates of vaporization, thereby permitting utilization of the favorable properties of these acids in SBB formulae.

PROPIONIC ACID

PSBB. When SBB was dissolved in 60% propionic acid (PSBB), a brown mixture was obtained that stained chromosomes a purple-brown. Details of chromonematic structure could be seen. The rapid evaporation of the solvent resulted in excessive precipitation of the dye, especially during the manipulations of smearing. Therefore, the following mixture was tried.

PSBB mixed in equal parts with LSBB. This mixture is a useful one. Mitotic figures in fresh scale meristems were well preserved and stained. Lactic acid alone in high concentrations did not preserve mitotic figures in previously unfixed root-tips. Mitotic figures from prefixed root-tips are equally well stained. Excessive precipitation of the dye seemed to be eliminated in this mixture but the preparations lasted for only a few days due to fading.

Propionic acid alone did not possess any outstanding characteristics. In combination with lactic acid it permitted the preservation of mitotic figures. In combination with formic acid it inhibited cytoplasmic staining as reported below.

ACETIC ACID

ASBB diluted with equal volume of water. When a 1% solution of SBB in glacial acetic acid (ASBB) was diluted with water and the precipitate separated, the red-brown filtrate stained nuclei and cytoplasm brown. The cytoplasm was less heavily stained. The prophase stages in prefixed root-tips were not as clearly differentiated as in the formic and propionic acid mixtures since the nuclear lymph also stained. The nuclei and chromosomes are swollen as one would expect in 50% acetic acid. While the uptake of the dye was moderately fast, warming the preparation as practised in the aceto-carminic technic, hastened the process. However, precipitation of the dye began at the edges of the coverslip as the acetic acid evaporated and the water concentration increased.

SBB is insoluble in hot formalin. When an equal volume of glacial acetic acid was added, the dye was dissolved. The color of the resulting solution was a deep blue. When used on prefixed root-tips, both nuclei and cytoplasm stained brown faintly, if at all, during

the time interval in which successful staining occurred with other mixtures. However, it is of pertinent interest to note that, in the presence of the aldehyde, the blue chromophoric state of the dye was obtained which did not stain nuclei or chromosomes.

FORMIC ACID

FSBB. SBB readily dissolved in 20% formic acid (FSBB) to yield a dark brown solution. In this solution the nuclei stained rapidly, changing from dark brown to black. The cytoplasm also stained but not as deeply. In the case of formic acid, the cytoplasmic staining may be encouraged by the slight amounts of HCHO present. The mordanting effect of formaldehyde upon cytoplasm when used with certain dyes in acid solution is well known (Zirkle, 1947). Formic acid could be considered as coexisting as an aldehyde because hydrogen instead of an alkyl group is linked to the carboxyl group.

FSBB mixed in equal parts with PSBB. From the results obtained with SBB in propionic acid alone, it was considered feasible to try a combination of formic and propionic acids in order to minimize cytoplasmic staining and still take advantage of the property of formic acid of dissolving SBB in the presence of a large concentration of water. Cytoplasmic staining was greatly inhibited by this mixture. Observations could be readily made upon relational coiling and chromonematic structure of the chromosomes at metaphase. However, this mixture had one of the technical objections encountered in 20% FSBB alone. The rapid evaporation of the solvents resulted in the precipitation of excess SBB. Whereas sealing would obviate some of the difficulty, there was not enough latitude for the necessary manipulation in preparing the smears.

FSBB, PSBB and 42.5% lactic acid mixed in equal parts. Earlier work had shown that lactic acid mounts withstood drying out under ordinary laboratory conditions. The volatility of formic and propionic acids seemed to be decreased in a mixture with lactic acid. While this mixture was found to be useful even though the cytoplasm stained slightly, improvement in the speed of staining and intensity was believed to be possible.

FSBB, LSBB, propionic acid and water mixed in equal parts. Several experiments were made in which the relative proportions of the constituents of the immediately preceding combination were altered. It was found that 0.5% SBB in a formic-propionic-lactic mixture containing approximately 50% water stained nuclei and chromosomes fast and with satisfactory intensity. Also at such

concentration the precipitation of the dye was minimized. The following mixture has given useful results on prefixed onion root-tips, as well as those of corn, bean and trillium: LSBB, propionic acid full-strength followed by FSBB and water added slowly with stirring, all in equal parts.

When this mixture was used as a fixative and stain on untreated root-tips and scale meristems, the internal structure of the chromosomes was preserved and stained with the diagrammatic clarity of a caricature. Interkinetic and early prophase nuclei were preserved and stained with simple, accentuated chromonematic structures requiring cautious interpretations.

TABLE 1. ACTION OF ACIDS UNSUITABLE FOR SBB FORMULAE

Acid	Solubility of SBB*	Color of Solution	Comment
Citric.....	+	Red-Brown	Brown stained chromosomes, badly swollen. Dye ppts., upon successive dilution. Staining of chromosomes slow in diluted solutions. Cytoplasm stains.
Tartaric (d or l)	+	Brown	Destruction of cellular content with gross alteration of nuclear structure. Ppt. of dye upon dilution. Chromosomes stained brown.
50% glyceric	—	—	Position and hydrophylic activity of hydroxyl groups seem to antagonize favorable action of carboxyl group upon solubility of SBB.
70% glycolic 50% glycolic	+ —	Red —	Chromosomes unstained. Diluted to 50%. Heavy ppt. Filtrate pale pink.

*Approximately 100 mg. of SBB dissolved in citric acid 16 g./10 ml. H₂O; in tartaric acid 13.2 g./10 ml. H₂O; in 50% glyceric acid 10 g.; in 70% glycolic acid 10 ml.

DISCUSSION

Sudan black B is a complex polyazo dye with the empirical formula C₂₉ H₂₂ N₆. It is a red brown powder and has been used as a general myelin, bacterial fat and leucocyte granule stain. See indices of Stain Technology, Leach (1938), Hartman (1940), Sheehan and Storey (1947). The solvents previously used for this dye were chiefly 70% ethyl alcohol, 50% diacetin (glycerol diacetate), 50% dioxane and ethylene glycol. Such solutions are deep blue.

Although the information derived from observations on various acid solutions of SBB is suggestive, it does not appear to be sufficiently extensive for the purpose of evaluating the relative importance and the effects produced by the length of the carbon chain and the number of hydroxyl and carboxyl groups upon useful chromosome staining. However, one relationship does seem to emerge, namely,

that the blue chromophoric state of SBB is incapable of staining nuclei and chromosomes.

Apparently the brown chromophoric state of SBB in the presence of an adequate water concentration which stains nuclei and chromosomes can be obtained only by certain organic solvent acids or mixtures thereof. Such solvent acids may produce a solution with a structure of the type most cautiously advanced in a footnote by Zirkle (1940).

The staining of nuclei and chromosomes usually involves salt formation in the presence of adequate amounts of water. SBB is inherently a hydrophobic dye. The rapid evaporation of certain solvent acids from some of the experimental preparations led to an excess of water and the resulting precipitation of SBB. The tendency of SBB to precipitate in the presence of excessive water, the required presence of adequate water to reduce high acid concentrations and to permit chromosome staining presented somewhat of a technical problem, especially, if the speed and intensity of staining were not to be sacrificed by lowered dye concentrations. The lactic-propionic-formic-acid mixtures serve as a temporary expedient for onion material.

The fixation images produced by the various solvent acids in high concentration upon previously unfixed material did not seem to differ except for the one produced by 85% lactic acid in which despiralization seemed to occur in all mitotic stages. Also, the spindle was not preserved. Despiralization seemed to occur as the result of the dissolving away of some nuclear and chromosomal constituents. An extreme condition was encountered in the rounding up of nuclear chromatin in numerous small spherules. This stage was also seen on badly deteriorated preparations which had been prefixed. Moderate despiralization and coincident swelling might be responsible for the accentuated chromonematic images observed.

While certain technics may preserve nuclear and cytoplasmic structures so that greatly esteemed fixation images are achieved, opportunities should not be overlooked to follow previously invisible, intermediate alterations of protoplasmic structures by phase microscopy during fixation under properly controlled conditions as advocated by Duryee (1937).³

Such precautions would tend to enhance the assessed validity of certain fixation images.

The novel use of SBB as a stain for chromosome smear prepara-

³Also unpublished material which Dr. W. R. Duryee kindly demonstrated and discussed with the writer last summer.

tions may suggest to other workers some additional uses. Bacterial and general plant histological staining have been indicated by certain preliminary experiments too fragmentary to report at this time.

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A RAPID TECHNIC FOR DEMONSTRATING MAST CELLS IN MOUSE SKIN¹

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ABSTRACT.—A simple rapid technic is described for demonstrating mast cells in mouse skin. The procedure requires about 60 minutes from time of specimen removal until permanent mounting. The steps comprise: (1) stretch-mounting of skin on a cardboard frame; (2) fixing and dehydrating in absolute ethanol for 15 minutes; (3) xylene washing for 10 minutes; (4) absolute ethanol washing for 10–15 minutes; (5) 3–4 minutes in a 0.1% aqueous solution of methylene blue; (6) dehydrating and differentiating in absolute ethanol; (7) clearing in xylene; (8) trimming and mounting. Cell counts may be made immediately, as well as high dry and oil immersion study of cytological detail of mast cells.

Larsson and Sylvén (1947) have described a method for estimating the number, distribution, and form of mast cells in mouse skin. The method involves a 48-hour fixation in various solutions of basic lead acetate and formaldehyde, followed by serial sectioning, staining in toluidine blue, and cell counting. But the technic is slow, and implies that a specialized and complicated fixation is essential. That this is not the case for skin mast cells is suggested by our own laboratory experience as well as by an examination of the older literature (as referred to in: Lee, 1928; Michels, 1938; Cowdry, 1943). We were therefore led to develop a mast cell technic which would (1) be rapid, (2) not require sectioning, (3) permit ready study of mast cell distribution simultaneously throughout the epidermis, dermis, and subcutaneous connective tissue, (4) permit cell counts. By combining features of technics dating back as far as Ehrlich, a simple satisfactory procedure fulfilling these desiderata was derived, and is described below.

PROCEDURE

Skin (e.g., from the abdominal area) is freed of hair, either by epilation with sodium sulfide or by shaving. The animal is killed by cervical fracture, and the skin is incised from axilla to groin on both sides, and across. The skin patch is retracted from the abdominal

¹The work reported in this paper is a portion of that being carried on under a grant from the National Advisory Cancer Council of the U. S. Public Health Service.

muscle layers by rapid tearing, and is stretched and pinned to a cardboard frame previously cut to appropriate dimensions (Plate 1, Fig. 1). A frame is used rather than a board so as to facilitate penetration of reagents from both sides.

The frame and attached tissue is immersed in:

- (1) *Absolute ethanol for a 15 minute fixation and dehydration period.*

In order to dissolve away soluble fats so that the later aqueous dye solution can more easily penetrate the tissue, the preparation is removed from the alcohol, drained, and immersed in:

- (2) *Xylene for 10 minutes.*

To remove the xylene, the preparation after draining is transferred to:

- (3) *Absolute ethanol for 10-15 minutes (same alcohol as was used for fixation).*

The preparation is then immersed in:

- (4) *A 0.1% solution of methylene blue chloride,² in distilled water for 3-4 minutes.*

In order to wash out the excess methylene blue and to dehydrate the specimen, the preparation is drained and washed in:

- (5) *Two changes of absolute ethanol for a total of 15 minutes.*

This period of time is adjusted depending on the differentiation desired. Ordinarily the mast cell granules in mouse abdominal skin retain a heavy blue-purple coloration, with other cells and tissues transparent or only faintly bluish. If washing is done excessively (i.e., for several hours) the mast cells show a progressive loss of coloration. In practice the appropriate degree of washing may be determined by occasional examinations under a dissecting microscope while the specimen is in its final absolute ethanol bath. If over-staining has occurred, slightly acidified alcohol washing will accelerate de-staining. When the desired degree of differentiation is achieved, the preparation is drained and transferred to:

- (6) *Xylene for clearing; which for mouse abdominal skin takes only a few minutes.*

The pins are removed from the now fairly rigid specimen, and the cardboard frame discarded. The specimen is cut down to include the area of interest, and mounted. In most of our preparations we have employed standard microscope slides and 24×50 mm. coverslips, having trimmed the tissue to about 20×45 mm. (Plate 1, Fig. 2). The tissue is mounted flat, with the outer skin surface down. To prevent air bubbles, it is essential that a generous amount of mounting medium be first placed on the slide. The specimen is carefully de-

²The lot used by the authors bore Certif. No. NA-27.



PLATE 1

FIG. 1.—Cardboard frame on which freshly excised abdominal skin patch is pinned prior to immersion in absolute ethanol fixation bath. Approx. Nat. Size.

FIG. 2.—Permanent mount of mouse skin prepared according to the method described.

FIG. 3.—Skin area from Fig. 2 under $6\times$ objective of dissecting microscope, to show general distribution of mast cells. Note hair tufts. Mag. $\times 250$ (approx.).

posited on this, with more medium added on top the specimen, followed by coverslip affixing. Balsam and polystyrene are suitable mounting media. If the tissue has shown any tendency to curl or wrinkle, a coin is placed on the coverslip after mounting, so as to exert a mild pressure during the setting process, although ordinarily the specimen flattens automatically and is ready for microscope study immediately after mounting.

COMMENT

Initially a number of tests were performed to determine whether sodium sulfide as used in epilation in any way affected the skin mast cells. Sulfide-epilated skin areas were compared with adjacent skin areas which had been subjected only to shaving, or to no epilatory treatment. In no instance did sodium sulfide induce observable changes in the mast cell picture.

Further preliminary studies revealed that during skin retraction almost all the subcutaneous connective tissue adheres to the dermal tissues. Fixed preparations of both skin and its underlying muscle layers revealed that a negligible number of mast cells remain associated with such muscle layers. It was concluded that the mast cell picture in retracted skin is substantially the true one.

The question of dye penetration is equally important. Focusing on mast cells at various optical depths, it may be established that methylene blue, as applied in this technic, penetrates the whole thickness of the mouse skin, and thus subjects all mast cells of the preparation to staining. In experimental conditions involving pieces of tissue thicker than mouse abdominal skin, the technic should be appropriately modified. For example, in studying the mast cell distribution in the skin area above and on the sides of an implanted tumor, it was necessary to dissect away at least three-fourths of the tumor mass, so as to ensure complete dye penetration, as well as sufficient transparency.

One of the virtues of the procedure here described is that the specimen is ready for study about an hour after removal from the body. Examination of the whole preparation under the dissecting microscope reveals the gross distribution of mast cells; and high resolution study of individual cells may be made under high dry and oil immersion objectives.

It is not the purpose of this note to discuss functional and chemical aspects of mast cell staining. Suffice it to say that the metachromatic granules are the only intracellular structures found to react with the methylene blue, and that the nuclear and cell outlines are discernible only by means of the stained granules (Plate 2).

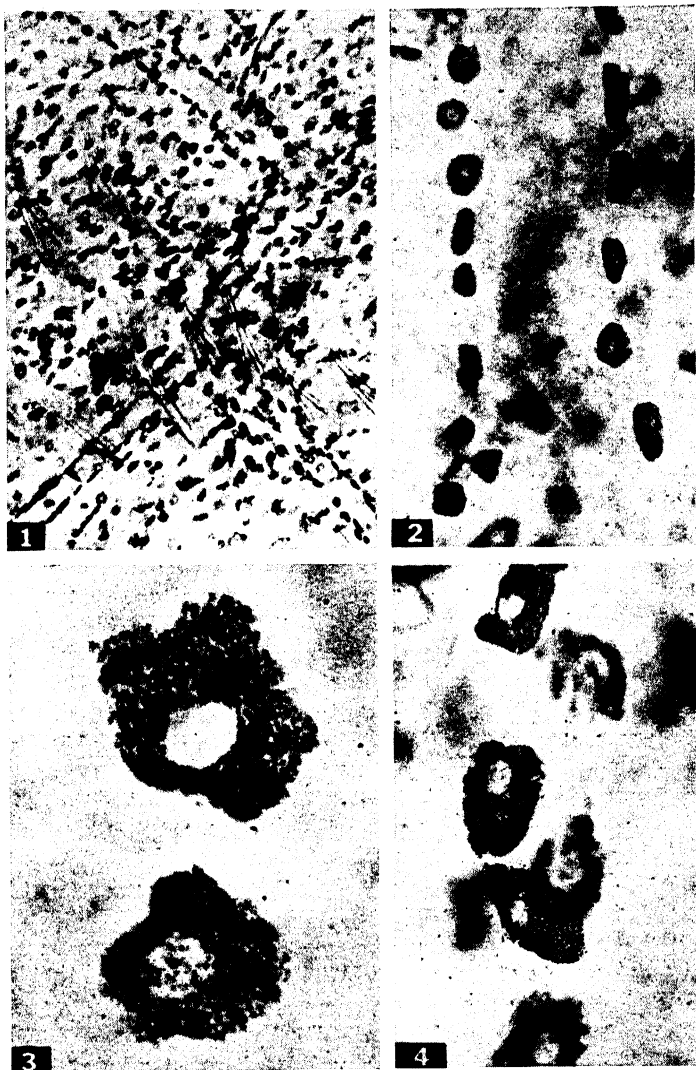


PLATE 2

FIG. 1.—Skin area under 16mm. objective to show location of mast cells in relation to blood channels. Note hair tufts. Mag. $\times 100$ (approx.).

FIG. 2.—Area from Fig. 1 to show enlarged blood channel with peripheral mast cells. Mag. $\times 400$ (approx.).

FIG. 3.—Mast cells from Fig. 1. Oil immersion. Mag. $\times 2400$ (approx.).

FIG. 4.—Mast cells from Fig. 1. High Dry. Mag. $\times 1200$ (approx.).

The technic could be useful both as a quick demonstration of skin mast cells for histology classes, and in the study of effects of various experimental agents on skin and connective tissue mast cells, especially when detailed cell counts are essential, or when the size and shape of cells or the form of the intracellular granules is of importance. With deep focusing a reliable count may be made of apparently all the mast cells of any specified area. The technic also reveals the location of mast cells in relation to such skin structures as vascular channels, hair follicles, areas of experimental injury, etc.

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NOTES ON TECHNIC

PONCEAU-FUCHSIN AS A ROUTINE COUNTERSTAIN

When making Kodachrome photomicrographs of sections stained with hematoxylin and eosin, it was found that the tissues stained by the eosin appeared in the photographs as a shade of orange rather than red. On the other hand it was observed that in the case of sections stained with acid fuchsin, the color of the tissues in the photomicrographs was the same as that in the original sections. Various stains were tried with a view to obtaining a simple counterstain for use with hematoxylin which would not only be more suitable than eosin for color photomicrography, but would also give better differentiation than the diffusely-staining acid fuchsin. The following stains were used: Biebrich scarlet; erythrosin; acid fuchsin; chromotrope 2R; Masson's ponceau-fuchsin mixture. The last stain gave the most satisfactory results and was used as follows:

1. Stain with hematoxylin (Ehrlich, Delafield or hemalum).
2. Leave sections in tap water till blue.
3. Stain with ponceau-fuchsin mixture for 2 minutes (1 part of stock solution diluted with 4 parts of distilled water.)
4. Wash in tap water for 1 minute.
5. Dehydrate, clear in xylene and mount in Clarite.

The ponceau-fuchsin stock solution is made up as follows: acid fuchsin, 0.35 g.; "ponceau de xyloidine", 0.7 g.; 1% glacial acetic acid, 100 ml. (Note. Ponceau de xyloidine is stated by its manufacturers to be identical with Ponceau 2R; Colour Index No. 79.)

The ponceau-fuchsin counterstain gives good differentiation between muscle fibers (which are stained bright red) and collagen connective tissue fibers (dull pink); it stains more crisply and brightly than eosin and is equally simple to use. It may therefore be employed with advantage as a routine counterstain to hematoxylin.

The stains used were supplied by Messrs. G. T. Gurr, London.

I wish to express my thanks to Professor Francis Davies for his interest and advice.—J. H. KUGLER, *Anatomy Department, University of Sheffield, England.*

DELAFIELD'S HEMATOXYLIN COMBINED WITH SAFRANIN FOR ANATOMICAL PREPARATIONS OF PLANT PARTS

A mixture of ripened Delafield's hematoxylin, made according to the original formula, and 1% safranin O is used for this staining

procedure. The safranin is prepared by mixing 1 gram of dry safranin O with 5 ml. of methyl alcohol. This mixture is then added to 95 ml. of distilled water. A combination in the proportion of 2 ml. of Delafield's hematoxylin and 38 ml. of aqueous safranin was found to be most satisfactory for paraffin sections of stems, leaves, and roots which had been killed in a fluid containing chromic acid. This results in a sharp contrast stain which differentiates cell walls especially well.

A mordanting period of one hour (or more) in 1% chromic acid followed by a thorough washing in tap water may be necessary for material killed in a mixture of formalin, acetic acid and alcohol before proceeding with staining.

Some animal tissues stain reasonably well in this combination. Sections of small intestine for example retain safranin in the mucous cells and the nucleoli. All else retains hematoxylin in varying degrees thus yielding fairly well differentiated preparations. Animal tissues apparently require a somewhat longer staining period (36 to 48 hours) than do plant tissues.

Procedure

1. Take slides down to water and stain in the combination (2 ml. Delafield's hematoxylin and 38 ml. 1% aqueous Safranin O) 12 to 24 hours (overnight is satisfactory).
2. Rinse in tap water.
3. Alcohols—30% and 70%—approximately one minute in each.
4. Differentiate in a saturated solution of picric acid in 70% alcohol—10 dips.
5. Rinse in tap water, 30% alcohol, and 70% alcohol.
6. Alcohol, 50% (made up to 0.1% sodium carbonate)—5 dips.
7. Alcohol, 95%—2 minutes.
8. Absolute alcohol—2 minutes.
9. Carbol-xylene—2 minutes.
10. Two changes of xylene—5 minutes in each.
11. Balsam.

The stains used were both products of the National Aniline Division, Allied Chemical and Dye Corporation.

Safranin O—Certification No. NS-19

Hematoxylin—Certification No. NH-2

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LABORATORY HINTS FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

JELLEY, EDWIN E. **Light microscopy.** *Anal. Chem.*, 21, 40-4. 1949.

This publication is a summary of papers presented at The Symposium of Light and Electron Microscopy held in Chicago, Illinois, June 10, 11 and 12, 1948. Included in the list are reports concerning the latest improvements in phase, reflecting, ultraviolet, infrared, fluorescence and phosphorescence microscopy. Other new developments mentioned are the variable focus condenser, combined vertical and concentric opaque illumination, hot stages for crystallographic microscopy and the optical dispersion staining method. A list of 150 references is given.—*G. C. Crossmon.*

PHOTOMICROGRAPHY

SHROPSHIRE, R. F. **Oblique photographic illuminator.** *Trans. Amer. Microsc. Soc.*, 66, 269-71. 1947.

Two prisms and a concave mirror have been arranged to bring the intense light beam from the lamp used for photomicrography to focus on the surface of an opaque object.—*Virgene Karanagh.*

MICROTECHNIC IN GENERAL

BURTON, C. J. **Electron microscopy.** *Anal. Chem.*, 21, 36-40. 1949.

The various techniques utilized in preparing specimens for examination with the electron microscope are discussed in this review. (159 references.)—*R. T. Whittenberger.*

COMER, J. J., and HAMM, F. A. **Modified silica replica technique.** *Anal. Chem.*, 21, 418-9. 1949.

A conical basket, made of 20-mil tungsten wire, is used as the heating unit for vaporizing silica onto microscopic specimens in high vacuum. Three methods are used. In the first silica flakes, which may be obtained by allowing some Ludox (colloidal silica dispersion) to evaporate on a glass slide, are packed in the tungsten basket. In the second method a clean tungsten filament (basket) is dipped four times in Ludox; the filament is allowed to dry before each successive dipping. On a third tungsten filament are hung 4 or 5 quartz fibers about 0.5 mm. in diameter. Three sublimations are carried out in the same vacuum. The relative lengths of time (seconds) required to obtain silica films of comparable thickness are: quartz fibers, 45; Ludox flakes, 15; filament dipped in Ludox, 10 or less. The silica films are alike in all respects.—*R. T. Whittenberger.*

HILLIER, J., KNAYSI, G., and BAKER, R. F. **New preparation techniques for the electron microscopy of bacteria.** *J. Bact.*, 56, 569-76. 1948.

The organisms are grown directly on the thin supporting membrane of the electron microscope mount in such a way that the organisms are not handled throughout the process except on the membrane. For solid media the following procedure is recommended: flood the surface with sterile distilled water rapidly; place as quickly as possible a drop of 0.5-1.0% collodion solution in amyl acetate on the surface to spread; permit the solvent to evaporate; remove the water with a pipette from under the membrane; check to see that the membrane is thin enough to be visible only at its edges; place a drop of a clean distilled water or saline suspension of bacteria on the membrane with a capillary pipette and withdraw the drop immediately; if the membrane is wet by the suspension, incubate

for 1-8 hours; examine under the light microscope to see when a suitable growth has occurred; select a suitable area and cut out the block of agar below it by cutting straight down with a sharp scalpel; lift the slab of agar with the membrane from the petri dish; slide it under a clean surface of distilled water where the membrane floats on the surface bearing the growth on its upper side; pick up the membrane on the carefully cleaned piece of 200 mesh screen; blot the water from the pores of the screen with hard filter paper; examine under light microscope to select a suitable area for the electron microscope; cut or punch out an area to fit the specimen holder. To apply this method to liquid media, form the membrane on sterile distilled water in a petri dish; evaporate the solvent; pipette off the distilled water; refloat the membrane on the culture media; proceed as above.—*Virgene Kavanagh.*

VAN DUIJN, C., JR. Substitutes for cedarwood oil. *The Microscope*, 7, 91-3. 1948.

The author suggests the use of anisole (methoxybenzene) and methyl benzoate for immersion with oil immersion objectives in place of cedar oil since they are volatile and evaporate from the slide without any residue. Anisole is preferred to methyl benzoate since it is nearer to the required refractive index and is more volatile. Some caution is necessary with uncovered preparations since these reagents tend to dissolve certain dyes such as pyronin and Victoria blue.—*G. C. Crossmon.*

DYES AND THEIR BIOLOGICAL USES

BRANCH, G. E. K., and TOLBERT, B. M. The spectra of some diaminotriphenylmethane dyes in concentrated sulfuric acid. *J. Amer. Chem. Soc.*, 71, 781-7. 1949.

The similarity of the spectrum of malachite green to that of triphenylmethyl ion indicated that, in sulfuric acid, malachite green exists as a triply charged ion with a structure similar to that of triphenylmethyl ion except for two dimethylammonium ion groups at the p and p' positions. With some phenylated dyes bands were observed in the neighborhood of 700 m μ . These bands were attributed to the addition of a proton to one of the terminal benzene rings, resulting in a carbonium ion compounded of a large number of mesomers, differing from each other in the location of the charge and in the distribution of the double bonds. This mesomeric ion could be considered as a triphenylmethyl ion in which the conjugate system was extended by three more double bonds. Bands attributed to this type of ion were mixed with bands due to the second ion and to ions of the simple triphenylmethyl type.—*R. T. Whittenberger.*

CLAYSON, D. H. F., and PIRIE, D. G. Observations on the proposed grading of ice cream by a methylene blue test. *J. Soc. Chem. Ind.*, 67, 147-50. 1948.

The methylene blue test recommended by the Public Health Laboratory Service for assessing the hygienic quality of ice cream was shown to be unreliable. The weakness of the test is due to the fact that organisms capable of surviving heat treatment considerably more drastic than that required by the Ice Cream Regulations can reduce methylene blue under the conditions of the test. The rate at which they do so depends to some extent on the ingredients used in the ice cream.—*R. T. Whittenberger.*

ANIMAL MICROTCHNIC

BARNES, J. M. The staining of the duodenal mucosa of rats following the injection of solutions of tannic acid. *Brit. J. Exptl. Path.*, 29, 495-500. 1948.

A striking black pigmentation of the duodenal mucosa develops within 7-10 days after a single subcutaneous injection of 1 ml. of 20% tannic acid into the white rat. The staining is due to a deposit of some iron-containing pigment—almost certainly iron tannate—on the adventitial membrane of the capillaries in the duodenal villi. The staining develops within 2-3 weeks to a maximum and then gradually fades over the next 6-10 weeks. Repeated injections of tannic acid intensify the deposition of the pigment. The feeding of iron in excess affects neither the rate of development nor the intensity of the staining. It will also develop in young rats on an "iron-free" diet. By-passing the duodenum by a

gastro-enterostomy will prevent its becoming stained. The phenomenon is discussed briefly, but no satisfactory explanation can be offered.—*J. A. Kennedy.*

BUCHER, GORDON E. *The anatomy of Monodontomerus dentipes Boh., an entomophagous chalcid.* *Canad. J. Res., sec. D, 26, 230-81. 1948.*

To homologize the structures of *Monodontomerus dentipes* Boh., a highly modified insect, with those of more generalized insects, the anatomy of adults was studied by the usual methods of serial sections. Dissections were made in a variety of liquids; the most useful were Ringer's solution, 70% alcohol, and a mixture of alcohol and glycerine. A 0.5% solution of acid fuchsin sometimes proved helpful for distinguishing structures. To study the skeleton, the following procedure is recommended: clean in 10% sodium hydroxide; bleach in a mixture of KClO_3 crystals, HCl , and alcohol; stain with acid fuchsin. To obtain serial sections, fix in Lebrun's modification of Carnoy's fixative, embed in celloidin, and stain in Ehrlich's hematoxylin and eosin or iron hematoxylin and erythrosin. The anatomy is described and illustrated in considerable detail.—*H. P. Riley.*

LEVADITI, J. C., LEPINE, P., and ANGIER, J. *Etude au moyen de la microscopie en fluorescence, des inclusions intro-cellulaires acidophiles provoquées par les virus de la rage, de la vaccine, de la maladie de Borna.* *Compt. Rend. Acad. Sci., 227, 1061. 1948.*

Paraffin sections, after having been passed down to water, are stained for thirty minutes in a solution of two parts thioflavine S to 1000 parts water. They are thus passed rapidly through absolute alcohol and toluene and mounted in a minimum amount of balsam. For photography of filters transmitting, 4000Å gave the best results for fluorescence and 5150Å for visible light.—*T. E. Weier.*

MARSHALL, A. H. E. *A method for the demonstration of reticulo-endothelial cells in paraffin sections.* *J. Path. and Bact., 60, 515-7. 1948.*

The following method allows reliable silver impregnation of reticulo-endothelial cells in paraffin sections of formalin-fixed materials: Cut sections 15-20 μ . Without mounting on slides, transfer directly to 2 changes of xylene. On removal of paraffin place sections first in absolute alcohol, then in 50% alcohol. Place sections in the following silver solution for 5-10 sec., prepared by adding, without shaking, 10% AgNO_3 to 2 ml. ammonia of sp. gr. 0.880 until there is a slight permanent turbidity. Transfer to 8% formalin in distilled water 2-3 min., moving the section about. Wash in distilled water. Tone in 0.5% gold chloride, wash, fix in 5% sodium thiosulfate, dehydrate, and mount in balsam. Optimum time in the silver solution is usually 7-8 sec. If an especially intense impregnation is desired, after reduction in formalin transfer for 5-10 sec. to a mixture of distilled water 10 ml., silver solution 3 ml. and reduce again. For nuclear staining, wash sections in 2 changes of distilled water after reduction in formalin. Place for 1 min. in strong Ag_2CO_3 solution (details unavailable). Transfer directly to 10% formalin and agitate. Wash, tone in gold chloride, and mount. The cell bodies and processes of reticulo-endothelial cells stain black. In nerve tissue astrocytes and oligodendroglial cells are impregnated. Myeloblasts are not impregnated. Reticulin fibers are usually not impregnated; neither are epithelial elements.—*S. H. Hutner.*

MAYHEW, R. L. *A method for using methylene blue and eosin as a stain for sections.* *Trans. Amer. Micr. Soc., 66, 398-9. 1947.*

Mallory's method of staining with methylene blue and eosin frequently understains with the eosin. The following modification has proved to be satisfactory: remove paraffin from sections fixed in Zenker's fluid and hydrate; stain in a solution of 1 g. methylene blue (National Aniline NA-11) and 1 g. borax in 100 ml. distilled water for 3-5 min.; wash off stain in distilled water and pass through 50 and 70% alcohol; differentiate in 85% alcohol to which a few drops of a 10% solution of resin in absolute alcohol has been added quickly; wash in 85% and 95% alcohol to stop destaining; counterstain in a solution of eosin (National Aniline NE-5) 0.5 g. in 100 ml. 95% alcohol for a few seconds. Slides mounted in euparal have retained their color for 16 years. Various brands of dye have been proved satisfactory.—*Virgene Kavanagh.*

RAMSBOTTOM, J. M., and STRANDINE, E. J. Comparative tenderness and identification of muscles in wholesale beef cuts. *Food Res.*, 13, 815-80. 1948.

Fifty of the larger muscles were dissected from the wholesale cuts of three U. S. Good beef carcasses. They were weighed, and their percentage of the total weight in each wholesale cut was recorded. Moisture, fat, and pH determinations were made. Tenderness was determined by committee rating, histological rating, and a shearing device.

Representative samples of both raw and cooked muscles were prepared for histological examination. Small pieces of the raw muscles were fixed in 10% formalin, dehydrated in alcohol, embedded in paraffin, and sectioned. Weigert's elastic connective tissue stain was used to show the distribution of the elastic connective tissue, while Mallory's triple connective tissue stain was used to demonstrate the collagenous connective tissue. From the cooked muscles larger samples of muscles (approximately $1\frac{1}{2} \times \frac{3}{4}$ inches) were sectioned on the freezing microtome and stained with gentian violet and Sudan III.

The beef muscles varied greatly in weight, moisture and fat content, pH, and tenderness. It is apparent from these data that the commercial practice of grouping muscles of similar tenderness should be extended, so that the occurrence of both tough and tender muscles within a roast or steak would be eliminated in so far as it is practical.—*William G. Walter.*

VALADE, PAUL. Recherches histochimiques sur les lipides de l'écorce cerebrale. *Compt. Rend. Acad. Sci.*, 227, 646-8. 1948.

The phosphatides and galactolipids in the brain are demonstrated by a modification of Ciaccio's method which depends upon the insolubility of the major portion of the nerve lipids in acetone and the increase in insolubility obtained through the use of cadmium salts. The method is fairly complicated and the original article should be consulted for details.—*T. E. Weier.*

VALLANCE-OWEN, J. The histological demonstration of glycogen in necropsy material. *J. Path. and Bact.*, 60, 32-7. 1948.

Formaldehyde (4%) is as efficient a fixative for glycogen as Bouin's fluid or absolute alcohol. There is only slight deterioration after 38 days in formaldehyde. This experimental study was carried out on rabbit liver, and the glycogen was stained with Best's carmine.—*S. H. Hutner.*

PLANT MICROTECHNIC

COTTRELL, HELEN J. Tetrazolium salt as a seed germination indicator. *Ann. Appl. Biol.*, 35, 129-31. 1948.

In an attempt to find a chemical test for seed germination that would be more rapid than the standard laboratory test, German workers during 1939-1945 tried 2:3:5-triphenyl tetrazolium chloride and 5-methyl-2:3-diphenyl tetrazolium chloride using maize. As information in England concerning other cereals and legumes was inadequate, an assessment of this method is made using the triphenyl tetrazolium salt and the following method: select seeds at random and steep in tap water for 18 hours; section longitudinally through the embryo; place one half of each in a 7 cm. Petri dish; pour a 1% solution of a tetrazolium salt over the seeds; soak in the dark for 4 hr. at 20° C.; wash with tap water; examine seeds for staining. Viable seeds will be stained. Agreement between this method and the standard germination tests is good in the majority of experiments. The time is reduced by this method but the technic is more difficult and the method is not well adapted to certain seeds.—*H. P. Riley.*

MICROÖRGANISMS

BAKER, H., and BLOOM, W. L. Further studies on the Gram stain. *J. Bact.*, 56, 387-90. 1948.

Escherichia coli, normally Gram-negative, became Gram-positive when a highly viscous solution of desoxyribonucleic acid was added to bacterial suspensions in either distilled water or saline. This Gram-positive reaction was washed away by five washes with distilled water but not by five washes with saline.—*Virgine Kavanagh.*

BAYLISS, M., GLICK, D., and SIEM, R. A. **Demonstration of phosphatases and lipases in bacteria and true fungi by staining methods and the effect of penicillin on phosphatase activity.** *J. Bact.*, 55, 307-16. 1948.

The Gomori methods for demonstrating acid and alkaline phosphatases and lipases were applied to several bacteria and fungi with considerable success. Alkaline phosphatase activity is demonstrated by precipitation of the phosphate liberated as the calcium salt and its subsequent conversion to cobalt phosphate and then to cobalt sulfide. Acid phosphatase activity is demonstrated by precipitation of phosphate liberated as the lead salt which is then converted to lead sulfide. Lipase activity frees high-weight fatty acids which are precipitated as the calcium salts, then converted to the lead which is demonstrated as the sulfide. —*Virgene Kavanagh.*

COOK, A. H., ELVIDGE, J. A., and HEILBRON, SIR IAN. **Fertilization, including chemotactic phenomena in the Fucaceae.** *Proc. Roy. Soc., Sec. B., London*, 135, 292-301. 1948.

Detailed instructions are given for methods of studying fertilization in *Fucus serratus*, *F. vesiculosus*, *F. spiralis*, and *Ascophyllum nodosum*. These instructions occupy three and one-half pages of text and cannot be condensed. —*T. E. Weier.*

CRUICKSHANK, J. C. **A simple method for testing dye sensitivity of *Brucella* species.** *J. Path. and Bact.*, 60, 328-9. 1948.

The following is a convenient method for testing the sensitivity to dyes of species of *Brucella*: Place strips of filter paper (Postlip 693) 6×0.5 cm. in Petri dishes and sterilize. Pick with sterile forceps and dip one end in a sterile solution of the dye. Dry in the 37° C. incubator overnight. Concentrations of dye solutions used are: thionin, 1:800; basic fuchsin, 1:200; methyl violet, 1:400; pyronin, 1:80; all National Aniline.

For use, lay the strips impregnated with each of the dyes parallel and equally spaced on the surface of a plate of liver agar. Melt a tube of the same medium (12 ml.), cool to 50° C., and pour on top. When the agar is set, dry the plate at 37° C. Add a milky suspension of known strains of *Brucella*, and streak them at a right angle to the filter paper strips, using a spreader 8 mm. in width. The plates, when dry, are incubated at 37° C. in 10% CO_2 . Six strains can be accommodated on one 10-cm. plate.

Sensitivity to a dye is indicated by inhibition of growth across a strip. —*S. H. Hutner.*

DOWDING, ELEANOR SILVER. **The spores of *Histoplasma*.** *Canad. J. Res., sec. E*, 26, 265-73. 1948.

As there is indirect evidence that *Histoplasma capsulatum* causes benign infection, it was thought advisable to study the spores of *Histoplasma* in their saprophytic mold phase and in their transition to the parasitic yeast phase. As the usual method of transferring the mycelium by means of an inoculating loop to the mounting medium disarranges the hyphae and scatters the spores of *Histoplasma*, the following method was used: lower a sterile cover slip over a Petri dish culture until it comes into contact with the aerial mycelium; to examine immediately, place cover slip with spores on a slide containing a drop of cotton blue and lactic acid; for cultural study, place cover slip on a hollow-ground slide containing water with or without nutrient broth or agar and seal with vaseline; leave cultures at room temperature or at 37° C. for about a week. Chlamydospores appear under transmitted light as dark globules because they are immersed in liquid drops, and on lowering the cover slip the drops are pressed out into smears containing conidia. To study the yeastlike phase it is necessary to insure that the spores cannot agglutinate and that they become wet. To bring this about: almost fill depression in hollow-ground slide with Sabouraud's medium; when agar has solidified place drop of plain broth on it; rim depression with vaseline; lower cover slip with spores down over the medium; warm just enough to melt vaseline; place preparation on piece of bent glass tubing on damp filter paper in bottom of Petri dish and keep at 37° C. for a week. The mold stage contains tuberculate spores which have extensions of the spore contents through openings in the wall. The yeastlike phase arises from the hyphae, conidia, or from the tuberculations of the large spores (chlamydospores). —*H. P. Riley.*

KNAYSI, G. Preliminary observations on germination of the spores of *Bacillus mycoides* in a nitrogen-free medium and certain properties of the transparent cells. *J. Bact.*, 55, 753-7. 1948.

The cytoplasm of the vegetative cells which develop on the nitrogen-free medium does not stain with methylene blue and becomes Gram-negative; the nuclei remain Gram-positive. The cells are highly permeable to neutral red.—*Virgine Kavanagh.*

HISTOCHEMISTRY

PRICE, J. M., MILLER, E. C., and MILLER, J. A. The intracellular distribution of protein, nucleic acids, riboflavin, and protein-bound aminoazo dye in the livers of rats fed *p*-dimethylaminoazobenzene. *J. Biol. Chem.*, 173, 343-58. 1948.

Rats were fed a synthetic basal diet modified to contain 0.5 or 20.0 mg. riboflavin per kilo with either no azo dye or 0.06% *p*-dimethylaminoazobenzene. Of rats fed the dye in low riboflavin diet 90 to 100% developed liver tumors after 4 months. The livers were fractionated and analyzed for protein, nucleic acids, riboflavin and protein-bound aminoazo dye. Ingestion of *p*-dimethylaminoazobenzene in the diet low in riboflavin increased the protein and desoxypentose-nucleic acid contents of the nuclear fraction by 37 and 29%, respectively. Ingestion of the carcinogen in diets containing either low or high levels of riboflavin reduced the protein content of the large granules by 35%, lowered the levels of pentosenucleic acid in the large and small granules by about 40%, and decreased the levels of riboflavin in the large granules and supernatant fluid by 45%. When *p*-dimethylaminoazobenzene was fed, each liver fraction contained protein-bound aminoazo dye. The highest concentrations were found in the small granules and supernatant fluid. Ingestion of a high level of riboflavin lowered the level of the bound dye in each fraction.—*J. E. Kindred.*

SINGER, M., and MORRISON, P. H. The influence of pH, dye, and salt concentration on the dye binding of unmodified and modified fibrin. *J. Biol. Chem.*, 175, 133-45. 1948.

The affinities of films of human fibrin for the acid dye, orange G (certified, purity of sample 89%), and the basic dye methylene blue (certified, purity of sample 84%), have been studied under controlled conditions of pH (2 to 10), ionic strength (0.01 to 0.15) and dye concentration (1 to 124×10^{-5} M). The degree of combination between fibrin and dye was a function of these variables. Treatment of fibrin by heat or formaldehyde resulted in an increase in its affinity for acid and basic dye and a simultaneous or subsequent alteration in its relative affinities for the two dyes. The second effect was correlated with changes in the isoelectric point of the treated fibrin as measured by electrophoretic migration. The maximum binding of anionic and cationic dye at low and high pH, respectively, was much less than the estimated number of charged groups and this binding capacity varied with the ionic strength and concentration of dye.—*J. E. Kindred.*

STAIN TECHNOLOGY

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THE DEMONSTRATION OF A NEW NERVE-CELL ORGANOID

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ABSTRACT.—A method is described for the demonstration of a new nerve-cell organoid—the binary spheroid systems (Baker bodies). Zenker-formol or acetic-osmium-bichromate materials are postchromed at 37°C. and embedded in paraffin, sectioned and mounted in the ordinary manner. The sections are colored in a 70% alcohol solution of Sudan black B, rinsed in 70% alcohol, counterstained in Mayer's carmalum and mounted in Farrant's medium. After examination the cover may be removed and the Sudan black extracted in 96% alcohol. The sections can then be restained by the azan method of Heidenhain, and other cytoplasmic inclusions can be correlated accurately to the functional states of the spheroid systems.

A system of binary spheroidal bodies in nerve cells (Baker, 1944) has been studied in both invertebrate neurones and vertebrate sympathetic ganglion cells (Thomas, 1947, 1948), and in Purkinje cells (unpublished). These bodies, originally revealed by Sudan black in frozen sections, have been observed in living neurones after vital staining, and by phase microscopy (Kempson, Thomas and Baker, 1948). They consist of a core of non-lipoidal chromophobe material in a lipid sheath, exhibit cyclic activity, and influence production of neurosecretory granules (of Scharrer, 1941).

Routine fixatives (such as Zenker-formol) and schedules for paraffin embedding may be employed in the new staining method. The spheroids may be stained with Sudan black, then decolorized with 96% alcohol, and finally restained with a trichrome stain such as the "Azan" stain of Heidenhain. Drawings or photographs of the same cells with both types of staining give, by comparison, the precise relationships between the spheroids and other structures.

The method above shows also the "Golgi zone" in many cells; for

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example, the epithelial cells of small intestine (rat) shown in Fig. 1. The constancy with which results are obtained gives the technic a considerable advantage over the capricious metallic impregnation methods wherein negative results are usually meaningless.

METHOD

Fixation and embedding.

1. Fix small pieces of tissues in either Zenker-formol, 10%, (Maximow's fluid) or Bensley's A.O.B. solution:

2.5% potassium bichromate (aq.) 8.00 ml.

2% osmium tetroxide 2.00 ml.

Glacial acetic acid 1 drop only

The formalin used in making the Zenker-formol should be neutralized with chalk.

Small pieces should be fixed for 24 hours in either fixative.

2. Rinse quickly in two changes of tap water (5 minutes).

3. Place in a saturated solution of potassium bichromate at 37°C. in an incubator for 3 days.

4. Wash in running water for 1-6 hours.

5. Dehydrate in alcohol, de-alcoholize in ligroin and embed in paraffin wax.

6. Sections are mounted on slides in the ordinary manner using Mayer's egg-albumen-glycerine adhesive.

7. When dry remove the wax in xylene, and take the slides down to 70% alcohol.

8. Shake the slide free from excess 70% alcohol and place it section downwards on a concave staining plate (Gurr, London). Carefully withdraw with a pipette some Sudan black solution from the surface layers of the stock (saturated) solution in 70% alcohol, being careful not to disturb the sediment of undissolved Sudan particles resting on the bottom. Run enough staining solution under the slide to cover the section and allow it to stain for 7 minutes. Any concave staining receptacle, for example, a large clock glass, can be used to provide the means for staining the section in the inverted position.

9. Quickly rinse the slide in 70% alcohol for 15 seconds to 1 minute. Agitate the slide vigorously.

10. Place in a jar of tap water and agitate the slide for 1 minute.

11. Stain the nuclei by immersion in Mayer's carmalum solution, 2 minutes.

12. Rinse in tap water.

13. Carefully dry round the section to remove excess water from the slide and mount in a drop of Farrant's medium. Place on a hot plate for 7-10 days to clear and harden thoroughly.

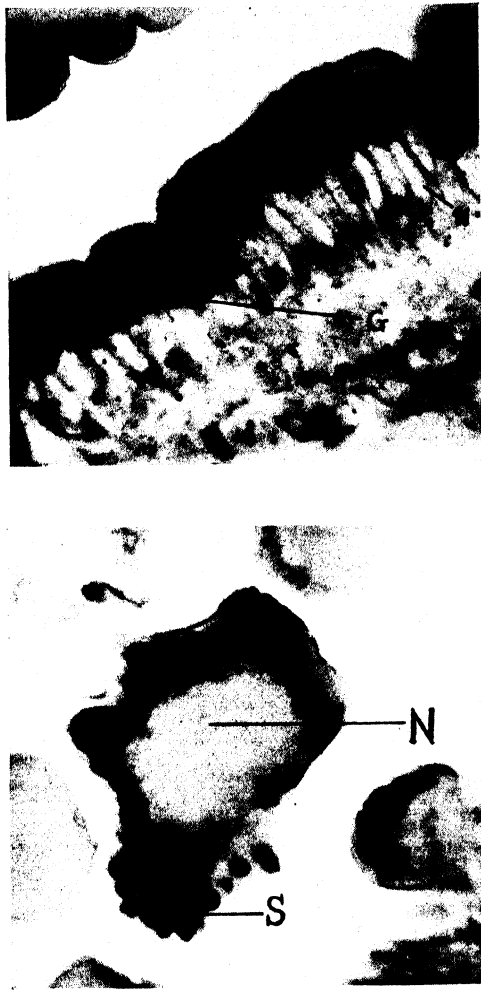


PLATE I

Photomicrographs taken with a Zeiss 2 mm. apochromat, n.a. 1.30, and a 10 \times compensating ocular.

1. (Top). Paraffin section of the small intestine of a rat. Zenker-formol fixation. Post-chromed. Sudan black technic. N—nucleus. G—Golgi Zone. $\times 1300$ (Approx.)

2. (bottom). Paraffin section of *Helix pomatia* cerebral ganglion. A.O.B. fixation. Sudan black technic. S—spheroid systems. N—nucleus. $\times 3000$ (Approx.)

Alternatively, Sudan preparations may be mounted in balsam as is described by Salthouse, 1943. This method works satisfactorily and gives a harder mount.

If it is desired to destain the preparations and to restain with a trichrome technic, step 11 should be omitted and Farrant's medium used for mounting. The cover glass may be removed readily by soaking the slide in water and the sections passed up to 96% alcohol. All traces of Sudan black will be removed rapidly in strong alcohol. The slide is then downgraded to water and staining commenced as for the trichrome method selected.

The most suitable Sudan black to use is that supplied by the British Drug Houses, London, England (Batch No. 404194). In solution it should be a distinctly carbon black color when a thin film of the solution is viewed with transmitted light (as when the bottle is shaken and the fluid running down the sides is examined). Solutions that are distinctly purplish or bluish black should be rejected as they are never so suitable. Dr. Baker informs me that the present sample of B.D.H. Sudan black as used in the Department of Zoology at Oxford dissolves to less than 0.4% w/v in 70% alcohol.

The ganglion cells of *Helix aspersa* or similar pulmonate proves an ideal tissue for studying the spheroid system of the nerve cell by the above method. Successful results are assured if these instructions are followed carefully. Fig. 2 is a photomicrograph of a *Helix* neurone prepared by this method. It is a striking fact that after decolorizing in strong alcohol and restaining the preparation with hematoxylin and eosin the characteristic spheroids cannot be seen, yet on reapplying the first method they are as obvious as before.

The reason why Sudan black B, a powerful coloring agent for lipoids, should continue to color materials (the spheroid pellicles) in sections of tissues which have been extracted by alcohol and benzene remains somewhat obscure. McManus (1946) has shown that a considerable variety of fatty substances in tissues do survive the ordinary embedding processes and can be stained subsequently by Sudan black. It is also certain that in the short period of staining allowed by the technic Sudan black will only color fatty substances (Lison, 1936).

Postchroming in some degree is nearly always necessary in order to secure good results with the above technic. In this connection Baker (1944) has shown that postchroming retains lipins in tissues and that this process forms an essential step in his acid hematin histochemical reaction (see also Cain, 1947). It seems possible that the phospholipids are held as insoluble chromolipin compounds and that these compounds retain the same affinity as the lipins themselves for dissolving Sudan black. When postchromed tissues are placed in

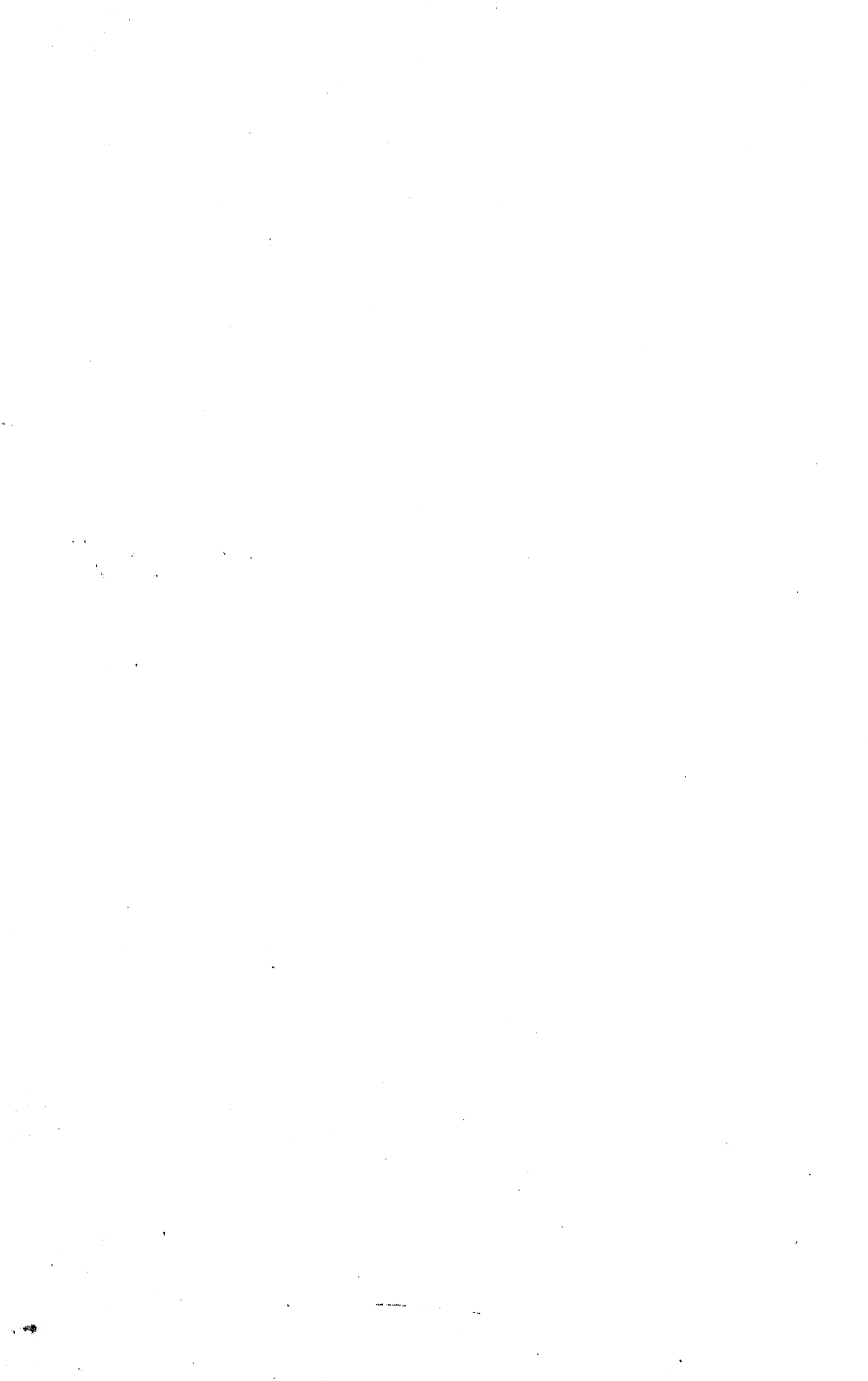
a 70% alcoholic solution of the coloring agent, the Sudan black deserts its alcoholic vehicle and dissolves in the chrome-lipid compound in which it has a greater solubility. The surrounding cytoplasm is left more or less colorless and a striking differentiation of the spheroids results.

Mr. E. H. Leach, of the Physiology Department, Oxford, has kindly shown me some sections of rat intestinal epithelium prepared by the above method which occasionally show rows of sudanophil droplets passing through the epithelium. These droplets are homogeneously colored and from their general distribution must be considered to be lipoids preserved and retained in the sections during fat absorption. In this connection Mr. A. J. Cain informs me that, according to his unpublished observations, the unsaturated triglycerides of ordinary fat droplets can be rendered insoluble after post-chroming, and are then colorable with Sudan black after paraffin embedding. Although penetration of fat-droplets by aqueous fluids is poor, nevertheless the outer shell of large droplets, which is retained and stained black, is far thicker than the sheath of a correspondingly large spheroid and cannot therefore be confused with it. As such shells do not stain with acid hematin it is probable that a chrome compound is not present. The unsaturated bonds have probably been oxidized, with a consequent rise in the melting point of the triglyceride.

I wish to thank Dr. J. R. Baker for his continued help and encouragement during the research which led up to the development of this technic.

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THIAZIN DYES IN SUPRAVITAL STAINING OF NERVE FIBERS

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ABSTRACT.—Supravital staining by thiazins of segments of small intestine and mesentery of young dogs was studied with reference to specificity for nervous tissue. Attempts to secure a purer form of methylene blue by alumina adsorption and alcohol elution of the commercial, medicinal dye yielded a product which appeared to be structurally different from the original dye. The treated dye had absorption maxima from 620 to 655 $m\mu$ in contrast with 665 for the untreated. Small nerve bundles were stained by the treated dye after 2 to 4 hours of immersion, but staining was always incomplete. Staining by untreated methylene blue was compared with that by the leucobase, thionol, methylene green, toluidine blue, new methylene blue and the azures. It was concluded that the specificity for nerve fibers resides mainly in the $=N(CH_3)_2Cl$ radical, although some specificity appears to be effected by the methyl groups on the trivalent nitrogen, since azure A (dimethyl) and azure C (monomethyl) stained weakly, but thionin did not. Methylene green showed some specificity but stained weakly. The leucobase was less active than the reoxidized dye obtained from it.

In a previous paper (1947) a technic for immersion staining of nerves with methylene blue was described in which the process could be sufficiently controlled to allow an analysis of factors which affect the reaction. The present paper is concerned with a further study of the staining reactions of interrelated thiazin dyes. The staining procedures used were the same as those described previously except where indicated.

Staining reactions were studied in the following manner. Three types of nerve elements were identified in segments of mesentery: (1) individual nerve fibers in the adventitia of the blood vessels, (2) small collections of non-myelinated fibers in and around the adventitia, and (3) larger bundles covered by a perineural sheath and coursing alongside the blood vessels. The type and intensity of staining of each were recorded as well as the reaction of the mesodermal tissue elements.

¹Publication No. 523.

In this study 217 segments of mesentery and intestine from 24 puppies or young adult dogs were used.

A. STAINING REACTIONS OF DIFFERENT SAMPLES OF METHYLENE BLUE

Nine samples have been used and their effectiveness graded from 0 to + + + +. Table 1 lists the results.

TABLE 1. STAINING REACTIONS OF SAMPLES OF METHYLENE BLUE

Dye	Date of manufacture	Effectiveness in staining nerve fibers
Grübler	1915	0
Schuchard	1925	+ +
Cert. No. NA-4 (Nat. Aniline)	1929	+
Cert. No. NA-8 (Nat. Aniline)	1933	+ +
Cert. No. NA-9 (Nat. Aniline)	1935	+ + + +
Cert. No. NA-11 (Nat. Aniline)	1936	+ + + +
No. 11437	1945	+ +
No. 11479	1945	+ + +
Cert. No. CA-23 (Coleman & Bell)	1947	+ + +

This indicates that not all methylene blue samples are equally effective in staining nerves. At first, this was thought to be due to aging and oxidation of the dye, but now appears more likely due to differences in dye composition. In all recent experiments, dyes of Certification No. NA-9 or NA-11 have been used.

B. THE PURIFICATION OF METHYLENE BLUE

In the previous paper it was pointed out that there were two phases in immersion staining with methylene blue. First there occurred staining of fat cells, smooth muscle, connective tissue and leucocytes. Later there was selective staining of fine nerve fibers and then bundles. It was suggested that the first or non-specific part of the process might be due to the presence of oxidation products such as the azure compounds. Extraction of azures was attempted to see if the specificity of staining could be improved. Two methods were tried.

(1) *Chloroform extraction.* Holmes (1927-1928) showed that commercial methylene blue contains 10% or more of the azures and described a method for extracting azure B and methylene violet. Solutions of the dye were made slightly alkaline (pH 7.35) with borate buffer and then extracted with chloroform. We modified this procedure by using a phosphate buffer to eliminate the physiologically toxic borate ion and three series of experiments were run: one at pH 5.6, one at 7.3 and another at 8.0. Three or four changes of

chloroform were used in each extraction. Care was taken to use the extracts only after all odor of chloroform had disappeared.

Solutions of purified methylene blue extracted at all three pH levels showed a loss of the specificity for nerve elements and an increased affinity for smooth muscle, connective tissue and endothelial cells, with the most vigorous staining occurring in alkaline solutions. Staining at a pH above 7 tended to produce the "azure type" of reaction in which there was limited staining of nerve fibers and bundles and intense staining of other elements. This suggested that azures were being formed in both controls and extracts. Chloroform extraction reduced the azure content but some azures may have developed, due to the alkalinity and temperature of the experiment (38°C.).

(2) *Chromatographic studies.* Since the results with chloroform extraction were not clear cut, it was decided to try to fractionate the commercial dye by the chromatographic adsorption technic. An adsorbing column was constructed of heavy pyrex tubing having an internal diameter of 3 cm. and a length of 60 cm. It was held in a vertical position by a clamp and ring stand and the lower end plugged with glass wool and a one-hole rubber stopper connected to a suction pump. A funnel was introduced at the top. Two types of adsorbents were tried. The first, activated carbon was found to be difficult to handle in that the dye could not readily be released from the adsorbing surface. Activated alumina (Aluminum Ore Company, Grade F, Mesh 20-40) was then tried and found more satisfactory.

The glass tube was filled with the alumina, a piece of filter paper placed on top of the granules, and the tube closed by the insertion of the funnel at the top. The suction pump was turned on and the column washed with either distilled water or phosphate buffer solutions as suggested by Rieman (1941). Samples of dye (0.25 to 0.30 g.) were dissolved in 100 ml. of either water, ethyl, or methyl alcohol and introduced into the top of the column. In some experiments the dye was sucked downward through the column and in others it was allowed to settle through without suction.

After passage of the dye, the column was washed with one or more liters of either distilled water or phosphate buffer (pH 5.6, 7.0 or 8.0) and then partially dried by sucking air through for 3 to 5 minutes. The alumina was then scraped out of the column in segments and each part placed in a beaker and covered with methyl alcohol, which proved to be an effective eluting agent. In some cases the alcohol was filtered off after 1 hour and in others after 48 hours, and the solution evaporated to dryness on an electric hot plate at 38°C. This

usually took about 24 hours. The dry dye sample was then scraped up and stored in clean vials.

Most samples were subjected to spectrophotometric study (by means of a Beckman spectrophotometer) and then tested for effectiveness in staining nerve elements.

It was found that activated alumina readily adsorbed the dye but without development of a chromatogram (stratification). Repeated washing with from 1 to 12 liters of distilled water or phosphate buffer failed to effect a separation of dye components, hence each column was arbitrarily divided into two or three segments and each segment eluted of its dye content.

Extracted dyes were found to vary in spectrographic absorption maxima from 620 to 655 $m\mu$ with little correlation between the level in the column and the maxima. All spectrographic curves had one thing in common; the adsorbed and eluted dye always showed a lower maximum than the control methylene blue (Cert. NA-9, absorption maximum 665 $m\mu$). In addition the absorption ratio (640/670 $m\mu$) of all extracts was higher than the control methylene blue. These facts indicated that adsorption on activated alumina changed the structure or composition of the dye.

The assumption of alteration in structure was further borne out in studies of immersion staining. The extracted dyes were observed to stain small nerve bundles after 2 to 4 hours but finer individual fibers did not appear at any time as they did in specimens stained with untreated methylene blue. In five of the extracts, tissue elements other than nerves stained intensely, thereby simulating the azure type of reaction (checked by control studies with azures B, A, and C). The remaining 11 samples were almost completely specific for nerve bundles with little or no staining of other elements.

This latter observation led to an analysis of factors responsible for the change in dye characteristics. Different solvents, eluting agents, and phosphate buffers of varying pH were tested and found relatively unimportant. One fact appeared significant, and that was the duration of contact of the alumina with the dye. Although the solubility of Al_2O_3 is very low (0.0001 per 100 ml.), it seemed that there might be enough alumina dissolved in the solution to form a complex with the dye. Such an effect was tested by adding 0.002 to 0.020% aluminum chloride to methylene blue solutions and examining the staining reactions. No effect was present.

It was concluded that activated alumina probably oxidized or in some other manner altered the molecular structure of the dye. The studies of Holmes and French (1926) indicated that two series of compounds may be derived from methylene blue. One series includes

the azures and thionin and is produced by acid oxidation of methylene blue. The other series includes azures B and A, methylene violet, methyl thionolin, thionolin, and thionol and is the result of alkaline oxidation. Experience with the staining reactions of the azures, thionin, and methylene violet indicated that these were not the same as the 11 samples of extracted dye that stained only nerve bundles. It appeared likely that the unknown derivative represented one or more of the thionols and an attempt was made to obtain these dyes for comparison.

Through the courtesy of Dr. L. Michaelis of the Rockefeller Institute for Medical Research a sample of thionol was obtained. This failed to stain any nerve elements although there was moderate staining of mesodermal elements. This represents a somewhat similar reaction to that of the unknown produced by alumina adsorption and suggests that it is either methyl thionolin, thionolin, or a mixture of the two. When these become available, their staining characteristics should be investigated.

Purification of methylene blue by chloroform extraction of alkaline solutions did not yield a dye which was more specific in staining nerve tissue. Adsorption on activated alumina produced a substance which was almost completely specific for nerve bundles but did not stain individual, isolated nerve fibers well. Neither process gave a product suitable for the usual type of supravital staining.

C. STAINING WITH THE REDUCED FORM OF METHYLENE BLUE

An attempt was made to use the reduced form of methylene blue (leucobase) to see if staining could be facilitated thereby. Two methods were used to obtain the leuco-base.

(1) *Reduction with hydrogen and platinum black.* Methylene blue was dissolved in 0.01% concentration in the substrata used previously for immersion staining, and to this was added about 1 g. of freshly prepared platinum black on asbestos fiber. Hydrogen gas was then bubbled through the fluid until it was completely decolorized. Tissue samples were added at this point and allowed to remain for $\frac{1}{2}$ to $1\frac{1}{2}$ hours. After this, they were transferred to plain oxygenated substrate for 2 to $2\frac{1}{2}$ hours more.

With this technic results were poor, and only occasional nerve fibers were stained. Other tissue components showed a moderate degree of staining. The lack of oxygenation of the tissues for 30 or more minutes apparently greatly disturbed the reaction.

(2) *Reduction with sodium hydrosulfite.* The leucobase was prepared also by adding a 2% solution of sodium hydrosulfite (NaHSO_2)

drop-by-drop to various concentrations of the dye dissolved in substrate until it was just decolorized. Solutions of 0.01 to 1.6% of dye were used. Precipitated sulfur was filtered out and then the tissue specimens added. Tissue was left in contact with this solution for 5 minutes to 2 hours and then placed in plain oxygenated substrate (without dye) for 2 hours. Staining was observed at half-hour intervals.

This procedure also yielded very poor results in that there was only pale staining of nerve fibers and heavy staining of all mesothelial elements.

In another group of tests the reduced dye was extracted from the aqueous phase with chloroform and then crystallized by evaporation. Long, yellow needles formed, which were rapidly oxidized by air back to the blue dye, and this material was used for staining. No significant difference from controls was noted.

It is concluded that the leucobase is much less effective in immersion staining than the oxidized form of the dye.

D. STAINING WITH METHYLENE GREEN, TOLUIDINE BLUE AND NEW METHYLENE BLUE

A series of tests was made to determine whether dyes other than pure methyl thiazine compounds would stain nerve fibers.

It was found that methylene green was slightly selective in staining individual nerve fibers and small bundles, which developed a pale green color. It also produced a diffuse green staining of mesodermal elements.

Toluidine blue and new methylene blue did not stain any of the nervous elements but stained the mesodermal structures with varying degrees of intensity.

COMMENT

The methods used to purify methylene blue did not yield any preparation which was as effective for staining nerve fibers as good batches of the commercial dye. Fig. 1 lists the different thiazines studied with their staining reactions and indicates that the non-specific part of the staining reaction is a property inherent in the thiazine group.

It appears that specificity for nerve fibers resides mainly in the $=N(CH_3)_2Cl$ radical, and when one or both methyl groups are removed (azure B and A), some of the specificity is lost. If the whole radical is removed (methylene violet) there is even further but not complete loss of specificity. Some of the specificity appears to be related to the trivalent N and its two methyl groups. There is still

FIG. 1.

FORMULAE and STAINING PROPERTIES of THIAZIN DYES				
	FORMULA	STRUCTURES STAINED		
		Individual nerve fibers	Nerve bundles (without epineurium)	Mesodermal elements
Thionine		0	0	++++
Azure C		0	+	++++
Azure A		0+	++	++++
Azure B		++	+++	++++
Methylene blue		++++	+++	++++
Methylene violet		0	++	+++
Thionol		0	0	++
Methylene green		+	+	+++
Toluidine blue		0	0	++++
New methylene blue		0	0	++

Intensity of staining graded from 0 to ++++

some staining of nerve bundles as long as one or two methyl groups are attached to this radical. When all four methyl groups from both radicles are absent, no staining of nerve fibers occurs.

The studies involving staining with leucomethylene blue were unsatisfactory because of the difficulty of maintaining the dye in the reduced form. The presence of air, oxygen, or tissue rapidly converted the dye to the oxidized form and it was impossible to make accurate observations regarding the actual staining process.

Staining by the immersion technic is still unsatisfactory for general neurologic research in that the reaction is not uniform, nerve bundles covered by epineureum stain hardly at all, and penetration of the dye is limited. Perfusion of the living animal as done by Feindel, Allison and Weddell (1948) may prove to be more satisfactory.

We wish to acknowledge our indebtedness to Professor Chester J. Farmer for his aid in the spectrophotometric determinations, and to the Biological Stain Commission for financial aid.

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A METHOD FOR DEMONSTRATING THE PRESENCE OF ALKALINE PHOSPHATASE AND GLYCOGEN IN THE SAME SECTION

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The distribution and relationships of alkaline phosphatase and glycogen has been the subject of study by a number of workers (e. g. Horowitz, 1942; Wislocki and Dempsey, 1945; Johnson and Bevelander, 1946; Pritchard, 1947), and attempts have been made to relate alkaline phosphatase activity with glycogen metabolism.

In some cases, the distribution of alkaline phosphatase and glycogen is similar, and there is difficulty in deciding whether certain cells contain both. Horowitz (1942) remarked upon this in his studies on fetal heads.

It seems that in general, the technical procedure has been to demonstrate the presence of alkaline phosphatase and glycogen in separate sections which are later compared, although Pritchard (1947) has apparently used a combined staining in his studies of the placenta, but gives no technical details.

While engaged in liver studies following feeding experiments on rabbits, I became interested in elaborating a technic which would show alkaline phosphatase and glycogen in the same section.

The following technic has been found to give good results, and depends upon the fact that absolute alcohol is a suitable fixative for both alkaline phosphatase and glycogen.

- (1) The tissue is fixed in absolute alcohol, cleared in xylene, embedded in paraffin at 56°C. and sections made.
- (2) The sections are treated by Kabat and Furth's modification (1941) of Gomori's technic for demonstrating alkaline phosphatase as far as the stage of treatment with ammonium sulphide.
- (3) After removal of the sections from the ammonium sulphide they are quickly rinsed in water, drained well and treated with freshly prepared Best's carmine for 10 minutes.
- (4) The sections are differentiated in Best's differentiating fluid, transferred to equal parts of ether and absolute alcohol—to remove the celloidin film, rinsed in absolute alcohol, cleared in xylene, and mounted in balsam.

RESULT

The presence of alkaline phosphatase is indicated by a black or deep brown deposit of cobalt sulphide, contrasting well with the

bright red appearance of the stained glycogen. Nuclei which do not give any phosphatase reaction are stained red, while those which are phosphatase positive¹ show a brownish-red color.

DISCUSSION

The combination of the two technics has to be made in the order given. Reversal of the procedure, i.e. treatment with Best's carmine prior to the demonstration of alkaline phosphatase, is unsuccessful as the glycogen stain will not be retained.

Ehrlich's hematoxylin is not used as a nuclear stain as is usual in Best's method, for the resulting dark blue stain does not allow a distinction to be made between nuclei that are phosphatase positive and those that are phosphatase negative.

Although Best's carmine is generally diluted immediately prior to use (Best's carmine, 2 vol.; concentrated ammonia, 2 vol.; methyl alcohol, 3 vol.), it will often be found that better results will be obtained without diluting the stain.

This method gives very satisfactory results with sections of rabbits' liver, the jet black bile capillaries contrasting well with the bright red glycogen stain. It is felt that the technic could be employed to advantage on other tissue material when there is doubt as to whether certain cells contain both glycogen and alkaline phosphatase. A certain degree of masking of weak cytoplasmic alkaline phosphatase by the stained glycogen is to be expected, and it will therefore be easier to note whether cells containing alkaline phosphatase are free from glycogen rather than the reverse.

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¹A positive nuclear reaction may be a genuine one or may be a pseudo-reaction due to a process of diffusion from neighboring structures of high phosphatase activity (Martin and Jacoby, 1948).

COMBINATION OF CERTAIN FLUORANE DERIVATIVE DYES WITH BACTERIAL CELLS AT DIFFERENT HYDROGEN ION CONCENTRATIONS¹

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While acid dyes have not been widely used for staining bacteria, several suggestions for their use have been advanced (Conn and Holmes, 1926; Maneval, 1941). In general these procedures call for adding an acid to the stain to intensify the combination of the dye with the bacterial cell. McCalla and Clark (1941) have demonstrated greater adsorption of acid dyes at lower pH levels. Conn and Holmes (1926) list a number of dyes derived from fluorane and compare their relative acidic tendencies, color intensities, and other properties. In a study of the influence of the acidic properties of dyes on their combination with bacterial cells at various hydrogen-ion concentrations, the data herein reported were obtained.

EXPERIMENTAL

Heavy suspensions of bacterial cells were obtained by growing the organisms in a peptone-yeast-extract broth. One-gallon bottles containing two liters of broth were inoculated with growth from Roux bottles and aerated vigorously with sterile air; after 24 hours incubation at 30°C. the cells were harvested by centrifugation. Following three washings with neutral distilled water, the cells were suspended in water in a concentration of from 5-30 billion cells per milliliter. All suspensions were stored at 3°C. and the experiments on dye uptake were carried out within 48 hours after harvesting. To test the uptake of the dyes at different hydrogen-ion concentrations, buffer mixtures of $M/100$ sodium acetate and acetic acid were prepared over the pH range 7.0 to 3.0. Throughout these studies all pH determinations were made with the glass electrode potentiometer. Ten milliliters of buffer were placed in a 20 ml. screw cap test tube and one milliliter of the heavy cell suspension added. It was necessary to check the pH value of the mixture after the addition of the cells because of their ability to combine with the buffer and cause a slight decrease in acidity. Four or five milliliters of a 0.003 to 0.001 molar solution of the dye being studied were then added. After

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¹Contribution No. 247, from Department of Bacteriology, Agricultural Experiment Station, Kansas State College.

mixing, the suspensions were allowed to stand at room temperature for 24 hours to reach equilibrium and then centrifuged. The concentration of the dye remaining in the supernatant fluid was then measured by spectrophotometric means. In all cases, control determinations were made on the dye and buffer in the absence of cells, to correct for any differences due to instability of the dye at the lower pH levels as observed by Holmes (1924). The dyes² chosen for this study, listed in order of their relative acidity as recorded by Conn and Holmes (1926), were eosin B, eosin Y, erythrosin B, and rose Bengal.

RESULTS

Fig. 1 shows the per cent of the total dye removed from solution at different hydrogen ion concentrations by cells of *Escherichia coli*. It may be observed that rose Bengal, the dye exhibiting the least acid

TABLE 1. PERCENT OF TOTAL DYE COMBINING WITH *BACILLUS SUBTILIS* CELLS AT DIFFERENT HYDROGEN ION CONCENTRATIONS

pH	Rose Bengal	Erythrosin B	Eosin Y	Eosin B
Suspension I: 2 billion cells per milliliter in 0.001 <i>M</i> dyes				
6.31	22	1	17	1
4.73	47	9	22	5
3.83	87	66	40	47
2.90	99	68	93	65
Suspension II: 5 billion cells per milliliter in 0.003 <i>M</i> dyes				
6.50	22	1	1	1
5.65	31	3	5	6
5.12	47	14	16	15
4.32	69	37	23	21
4.05	83	57	58	38
3.45	89	59	74	43

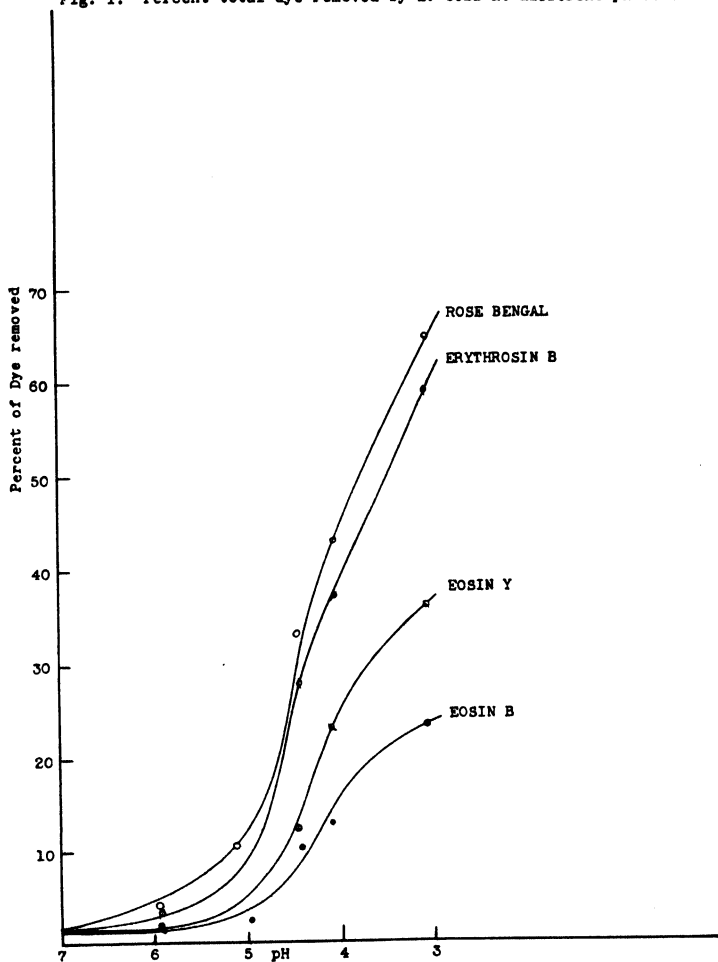
tendency, combined most readily with the cells at all pH levels. Also, the lower the pH level the more readily this dye combined with *E. coli* cells. In general, all dyes studied combined with the bacterial cells in the reverse order of their acidic ionization at all pH levels where measurements were made. Little significance should be attached to the absolute values recorded, since such values will vary with the concentration of cells and the concentration of dye.

In Table 1 are given the percentages of the initial dye combining with *Bacillus subtilis* cells in two experiments in which four different dyes were employed and the ratio between the concentration of cells and dyes varied in the two experiments. The relative position of the different dyes at the different pH levels remained remarkably constant.

²All dyes were certified dyes manufactured by National Aniline and Chemical Co.

A summary of data obtained in experiments with four other common bacterial types and the same dyes are recorded in Table 2. Again the order of the removal of dye is related inversely to the acidic character of the dye while combination of dye with cells at different

Fig. I. Percent total dye removed by *E. coli* at different pH levels.



pH levels is related directly to the hydrogen ion concentration. Again no comparison should be made between the different organisms since the concentrations of cells varied in the different experiments. The relative abilities of different bacteria to take up dyes might be

expected to vary with size, surface, chemical composition, ionization of cellular constituents, etc., none of which was taken into consideration in this study.

TABLE 2. PERCENT OF TOTAL DYE REMOVED BY CELLS OF *E. COLI*, *PROTEUS* SP., *LACTOBACILLUS* SP., AND *SARCINA* SP. AT DIFFERENT HYDROGEN ION CONCENTRATIONS

pH	Rose Bengal	Erythrosin B	Eosin Y	Eosin B
(Escherichia coli: 20 billion cells per milliliter in 0.003 M dyes)				
6.80	1	1	1	1
5.94	3	1	1	1
5.15	10	2	3	1
4.55	33	28	11	10
4.13	43	37	23	12
3.12	65	59	36	23
(Sarcina sp: 15 billion cells per milliliter in 0.003 M dyes)				
6.70	1	1	1	1
5.65	1	1	1	1
5.00	21	5	2	3
4.60	40	23	9	17
4.05	49	27	32	18
3.30	94	45	44	21
(Lactobacillus sp: 13 billion cells per milliliter in 0.002 M dyes)				
7.00	14	2	1	8
5.42	26	9	1	16
4.49	57	40	3	28
3.62	99	98	75	69
2.92	99	99	90	85
(Proteus sp: 19 billion cells per milliliter in 0.001 M dyes)				
7.18	22	11	3	2
5.41	49	28	3	16
4.53	77	69	20	36
3.82	95	97	58	73
2.79	99	99	81	95

It is a well established fact that bacterial cells of most, if not all, species carry a negative charge, which decreases as the hydrogen ion concentration of the suspending medium is increased. This over-all negative charge of the cell would tend to repel particles or ions of like charge, thus tending to prevent their combination with structures of the cell having a positive charge, such as free amino groups or other similar polar groupings in or near the cell surface. These dyes of the fluorane series possess the same general type of molecular structure, yet differ in degree of ionization as acids (Conn and Holmes, 1926). In such a series, the dye with the weakest negative charge (lowest acidic ionization) would be expected to exhibit the least repulsion by the over-all negative charge of a bacterial cell, and a weakly ionized dye should combine more readily with the cell than a more

strongly acid dye. Since the charge on the bacterial cells becomes less as the hydrogen ion concentration of the cell-dye mixture is increased, lowering the pH of such a mixture should facilitate combination of acid dyes with the cell. The results obtained in the present study tend to substantiate the concept that a suspension of bacterial cells in a solution of acid dyes behaves in the theoretical manner suggested.

Additional knowledge of the mechanisms by which dyes combine with the bacterial cell might yield valuable information concerning staining processes, and also help explain the uptake of nutrient materials by the living cell. This study suggests the urgent need for more exact information, particularly on the following points: The influence of various ions carrying an opposite charge to that of the chromophore ion upon the ionization of dyes; the over-all electrical charge of bacterial cells at different pH levels as determined by electrophoresis; and the combining capacities of dyes with bacterial cells at various hydrogen ion concentrations. Further studies along these lines are underway in this laboratory.

SUMMARY

Quantitative determinations of the amount of dye combining with cells of five common bacterial species at different pH levels have been made using four fluorane derivatives, eosin B, eosin Y, erythrosin B, and rose Bengal. Rose Bengal, the least acid of these dyes, combines most readily with the cells at hydrogen ion concentrations from pH 7.0 to 3.0. Adsorption of the dyes in this series was in general inversely related to the degree of acidic ionization of the dye molecule. All dyes combined with the cells more readily at the lower pH levels. A brief discussion of the theoretical implications in staining is given.

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A SIMPLIFIED METHOD FOR THE DOUBLE EMBEDDING OF TISSUE

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One of the major advantages of the paraffin method lies in the ease with which either continuous or interrupted serial sections may be prepared. Similarly the absence of distortion represents a very real advantage for the celloidin technic. Brown (1948) has recently remarked that double embedding methods may often be more suitable than either alone, but the technic he has outlined, which is similar to one we have used for some years, possesses the disadvantage that ribboning is impossible.

Because a projected research problem involved the preparation of serial sections from a large number of rat brains, we attempted to modify the double embedding technic to make ribboning possible. We found that reducing the amount of celloidin in the tissue by the complete elimination of one step greatly improved the technic. With the method as outlined below we find it possible to cut blocks (dry) routinely on a rotary type of paraffin microtome at thicknesses varying at least from 5 to 50 μ . All of these have ribboned well. Albumen has not proved satisfactory for affixing the sections to the slides but the Masson gelatin technic (Lillie, 1948) with the use of formaldehyde vapors for hardening the gelatin has proved adequate even for sections as large as sagittal sections through an entire rat brain. However, we find the use of fresh gelatin solutions necessary. In order to decrease wrinkling of sections we have found it advantageous to remove the slides from the warming plate as soon as the sections have spread, allow a few minutes for cooling and then drain off the excess gelatin solution. Then the sections are blotted down, flattened with filter paper and placed in the formaldehyde vapors. Among the stains in routine use with us are hematoxylin-eosin, cresyl echt violet, and thionin.

The following timing schedule is suitable for whole rat brains but it can be considerably altered with smaller masses of tissue:

1. Place the formalin fixed brains in 95% alcohol without washing where they remain for one week with two changes of the solution.
2. Transfer to 95% alcohol and ethyl ether (equal parts of each) for one week with two changes.

3. Place in a 3% solution of nitrocellulose in 95% alcohol and ether for one week. [We find it convenient to make up stock solution of 20% as given by Bensley, (1941) and dilute as needed].
4. Allow to remain in a 6% solution of nitrocellulose for one week.
5. Remove block of tissue from nitrocellulose, allow excess to drip off but do not allow surface to become hardened, and place in chloroform overnight.
6. Place in benzene for 48-72 hours with at least three changes.
7. Infiltrate with paraffin for about eight hours. We begin with about 48° paraffin with successive baths in slightly higher melting point paraffin and embed in 53°-55° paraffin. 8 hours.
8. Block and embed as if only in paraffin.

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A FIVE-HOUR VARIANT OF GOMORI'S METHENAMINE SILVER METHOD FOR ARGENTAFFIN CELLS

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ABSTRACT.—Gomori's methenamine silver method for argentaffin cells has been modified and considerably accelerated by almost doubling the silver concentration and raising the incubation temperature to 60°C. Argentaffin cells are selectively impregnated in 3 to 4 hours and the background remains relatively clear up to 4 hours. The contrasts are clearer than with the ammoniacal silver methods of Masson and of Gluckmann.

In a series of comparisons of the Masson silver method as modified by us¹ with Gomori's² recent methenamine silver technic, it was observed in the latter that irregularities in the rate of blackening of argentaffin cells seemed to correlate with temperature variations in the 37°C. incubator employed. As it appeared that it might be possible to shorten the Gomori method so that it could be completed on the same day, it was decided to try incubation in the paraffin oven at about 60°C. and at the same time to almost double the silver concentration.

It was found that very satisfactory demonstration of argentaffin cells in formalin-fixed paraffin sections could be obtained in 3–4 hours at 58°–62°C. When the temperature was raised to 65°C. on one series of sections, impregnation of argentaffin cells was more rapid but silver precipitated very diffusely over the slides. Hence, no further trials were made at this higher temperature.

The material used at this stage was human material removed surgically from stomach, duodenum, ileum, appendix, and colon, and fixed routinely in more or less acid formalin of about 10% dilution. Observations were then extended to stomach and intestine of rabbits, rats, guinea pigs and one dog, fixed in buffered neutral aqueous 10% formalin, as well as in other fluids. The materials fixed in other solutions gave quite variable results and will be included in a later study.

¹Laskey, A. and Greco, J. 1948. Comparative study of argentaffin cells of human appendix using modified Schmorl and Masson technics. *Arch. Path.*, **46**, 83.

²Gomori, G. 1948. Chemical character of the enterochromaffin cells. *Arch. Path.*, **45**, 48,

For material fixed in neutral formalin, the following procedure can be recommended.

Solutions:

Stock methenamine silver solution: Dissolve 3 g. methenamine (hexamethylene tetramine) in 100 ml. distilled water. Add 5 ml. 5% aqueous silver nitrate. This solution can be stored in a cool dark place for months.

Working solution: To 30 ml. stock methenamine silver solution add 8 ml. Holmes' pH 7.8 borate buffer.³

Glassware: The Coplin jars should be chemically clean. Previous silver mirror deposits should be removed with concentrated nitric acid.

Technic:

1. Treat deparaffinized sections with Weigert's iodine solution (I:KI:H₂O = 1:2:100)⁴, 10 minutes.
2. Bleach with 5% sodium thiosulfate (Na₂S₂O₃ · 5H₂O), 2 minutes.
3. Wash in running water, 10 minutes.
4. Rinse in 2 changes of distilled water.
5. Place sections in Coplin jars containing the buffered methenamine silver solution at room temperature and put in 60°C. (paraffin) oven for 3–3½ hours.
6. Rinse in distilled water.
7. Tone in 0.1% gold chloride (HAuCl₄), 10 minutes.
8. Rinse in distilled water.
9. Fix in 5% aqueous sodium thiosulfate, 2 minutes.
10. Wash in running water, 5 minutes.
11. Counterstain with 0.1% safranin O in 0.1% acetic acid, 5 minutes.
12. Dehydrate with acetone, clear in xylene, and mount as usual.

Gomori's directions called for 25 ml. of methenamine silver solution, 25 ml. of distilled water and 5 ml. of a borate buffer in which M/5 borax was used. During the earlier part of this work this buffer of Gomori's was used. It was soon found that the M/5 borax crystallized out on standing. It was then decided to substitute Holmes' borate buffers³. Usually Holmes' pH 7.8 buffer was used. Although higher pH levels gave more rapid impregnation, results were not as selective, as Gomori already noted, and the silver tended to precipi-

³See p. 265 in Lillie, R. D. 1948. *Histopathologic Technic*. The Blakiston Co., Philadelphia.

⁴Gomori (personal communication) has emended the technic, and uses Gram's iodine (I:KI:H₂O = 1:2:300).

tate diffusely over the slides. The molar proportion of buffer to silver complex was calculated to be nearly the same as in Gomori's final mixture, but the silver concentration in our final solution was slightly less than twice as high.

In the experimental use of this method incubation times were varied by half hour intervals for $1\frac{1}{2}$ to $4\frac{1}{2}$ hours. At 3 to $3\frac{1}{2}$ hours impregnation was optimal. Argentaffin cells were well blackened and appeared in as great numbers as with longer impregnations. Coarse connective tissue of the submucosa sometimes showed a variable amount of blackening at this time. In 2 to $2\frac{1}{2}$ hours' incubation, partial blackening of argentaffin cells in reduced numbers was seen. At $1\frac{1}{2}$ hours, only cellulose was black. At longer intervals than $3\frac{1}{2}$ hours, besides the connective tissue, the granules of eosinophil leucocytes, nuclei, smooth muscle and surface epithelium became blackened. By $4\frac{1}{2}$ hours a silver mirror had begun to appear on the sides of the slides and Coplin jars.

Gomori's regressive differentiation of over-silvered preparations with weak acidified iron alum solution did not give particularly satisfactory results with our variant. With the shorter incubation periods used in this new technic, it is more practical to repeat the procedure with a briefer silvering time on duplicate sections.

DISCUSSION

This technic in our hands gives sharper differentiation of argentaffin cells with less silvering of background than the Masson technic. The shorter incubation at higher temperatures in our procedure gives us results comparable with Gomori's 12-48 hour, 35-45° technic. Gluckmann⁵ used higher temperatures in the Masson technic and reduced the incubation period to 5 to 10 minutes. We found that Gluckmann's technic gave no better differentiation of the argentaffin cells than the longer original Masson method.

⁵Gluckmann, F. 1947. La détection rapide des cellules chromaffines intestinales par les complexes ammoniacaux d'argent à chaud. C. R. Acad. Sci., 224, 295.

WHOLE MOUNTS OF FLAT AND ROUND WORMS FOR MORPHOLOGICAL STUDIES

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ABSTRACT.—A method for making permanent whole mounts of flat and round worms is described. The specimens are mounted in a drop of acid fuchsin lacto-phenol on a slide and warmed for 6 hours at 60°C. The acid fuchsin is replaced by light cotton-blue (anilin blue, W. S.) in lacto-phenol, till the desired contrast is obtained. After this, the forms are mounted in pure lacto-phenol, using the coverglass. The margin of the coverglass is sealed with the sealing media devised by Dade and Waller (equal parts of damar balsam and beeswax).

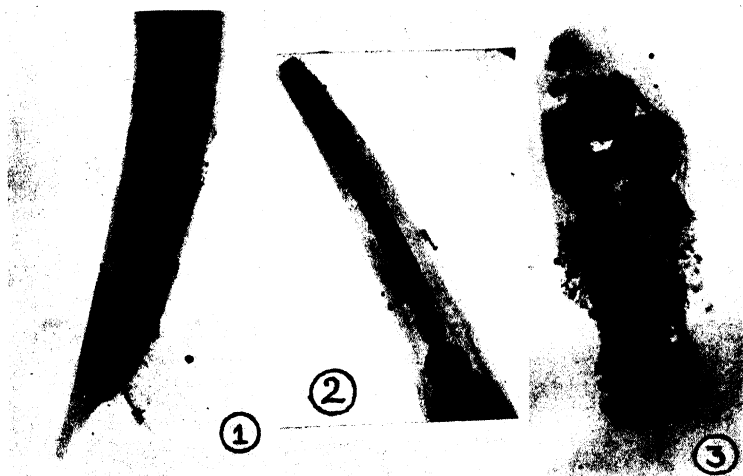


FIG. 1.—Nematode from the rectum of Pigeon. $\times 65$. Penial setae are clearly seen.

FIG. 2.—Nematode from the rectum of frog. $\times 64$. Anterior end showing the pharyngeal bulb.

FIG. 3.—Trematode from the rectum of frog. $\times 61$. The uterus with eggs, the testes, vitellarium and the genital aperture are clearly seen.

In the course of studies on some nematode parasites of plants, the need for staining and clearing whole mounts for morphological study

was keenly felt. It was also desirable to make such slides permanent. The usual methods of staining and dehydrating did not give the desired result on account of shrinkage.

The following method was therefore adopted for staining and clearing the material, a process which has been used by Dr. M. J. Thirumalchar for staining fungus material. The specimens were mounted in a drop of water on a slide. The water was drained off and a drop of acid fuchsin in lacto-phenol added (acid fuchsin added to lacto-phenol till a cherry red color was obtained). The slide was then warmed on a hot plate to about 60°C. for nearly six hours. After this, the acid fuchsin lacto-phenol was drained off, a dilute solution of cotton-blue (anilin blue W. S.) in lacto-phenol was added and the slide was slightly warmed. Observation revealed that the acid fuchsin was replaced by cotton-blue in some of the tissues while being retained in others. When the desired amount of differentiation was obtained, the material was mounted in pure lacto-phenol using a coverglass. For making the slides permanent, the margins of the coverglass were sealed with the sealing medium (devised by Dade and Waller¹) using a hot spatula. The sealing medium, composed of damar balsam and beeswax, has been found to be immiscible with lacto-phenol, and soon solidifies into a hard crust, rendering the slide permanent.

The same staining technic was employed for the following forms with very good results; nematodes present in the alimentary canal of frogs and Calotes; trematodes in the frog alimentary canal, and fish liver; tape-worms present in the pigeon intestine. It was observed in the case of nematodes that the external apertures and penial setae (Fig. 1) were very clear after staining. The internal organs such as the alimentary canal were also stained and the details of the structures could be clearly seen (Fig. 2). In case of trematodes, it was found that the forms with very thick body wall were poorly differentiated, because of the uniform staining. In forms with thinner body wall all the internal structures were clearly brought out (Fig. 3). The same method was also tried on plankton forms previously fixed in formalin. It was found that the acid fuchsin lacto-phenol method gave very good results and deserves a trial on similar forms.

In this connection I wish to thank Dr. L. S. Ramaswami, Professor of Zoology for his kind encouragement and also Dr. M. J. Thirumalchar for his kind help.

¹Dade, H. A., and Waller, S. Mycol. Pap. Commonw. Inst., 27. 1949.

STAINING OF PLANT MATERIALS CLEARED IN NaOH¹

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ABSTRACT.—Stains are listed which have proved suitable for staining the epidermis, mesophyll, and sclerenchyma and tracheary elements, respectively, of cleared leaf material of *Mouriri* and *Linociera*. Too rapid leaching is avoided by overstaining high in the dehydration series, destaining briefly in the same solvent, and moving through to xylene. Twenty to thirty minutes staining time is generally sufficient. Concentrations and solvents can be varied widely. If destained too much, the material can usually be replaced in the dye with no ill effects. A double stain schedule (Bonnett) of five to ten minutes in 1% Bismarck brown Y in 95% alcohol followed by one to two minutes in 1% fast green FCF in 100% alcohol may be advantageous for thin-walled cells in thick material. It may be preferable to treat thinner material with tannic-acid-iron-chloride followed by safranin (Foster). The effects of bleaches and clearing compounds other than NaOH on staining have not been investigated; however, Dr. Bonnett finds that lactic acid used after NaOH improves clearing and also improves the staining of his combination (above). Mordants can doubtless be used to advantage.

One of the simplest and best means of preparing plant material for the three-dimensional study of cell size, shape, and arrangement is that of clearing, done by bleaching or removing the pigments with suitable reagents. Although this method has long been known and used, its possibilities are not yet all exploited.

Cleared objects are often thin and transparent enough to be satisfactorily viewed when immersed in water or alcohol. In these media small veins and other details are usually readily visible, and no stains are needed. Frequently, however, the material is so opaque even after clearing that it must be run up into xylene for sufficient transparency. When this is done the increased refractive index of the medium renders many details of structure almost invisible, and a stain becomes desirable. Staining may also be needed to bring out thin-walled cells which are not easily seen even when in water.

Prolonged exposure to NaOH with the accompanying removal of many pigments and other substances alters the chemistry of the

¹For an account of this clearing method, see Foster (1949, p. 216–217).

plant tissues. The result is that many structures no longer have the affinities they once had for certain dyes, and most dyes tend to leach out rapidly when in water or alcohol. Thus stains cannot be relied upon to act as they would on uncleared material. This is apparently true to a lesser extent of materials cleared in other reagents, particularly those which are simply bleaching compounds. Evidently little exploration of this general subject has been done, although Stevens (1916) and Gabriel and Pellisier (1936) each give instructions for the use of two dyes with cleared material, and Stebbins (1938) does likewise for a single stain.

Using whole leaf-material of *Mouriri* (Melastomaceae) and *Lino-ciera* (Oleaceae) cleared in NaOH, over 20 different stains were tried in an effort to determine which could be used for bringing out the epidermis, mesophyll, and sclerenchyma and tracheary elements, respectively. The best of these are listed below, in approximate order of their successfulness.

Epidermis

1. ZnCl_2 -tannic-acid-iron-alum. Sometimes more effective without the ZnCl_2 ; also when following treatment with Stockwell's fluid (see Johansen, 1940, p. 85). Use 1% tannic acid; otherwise follow Scharman (1943).
2. Harris' hematoxylin, stained to taste, blued with tap water; not destained.
3. Delafield's hematoxylin, ca. 20% aqueous. As with Harris'.
4. Aniline blue, ca. 1% in 95% ethyl alcohol. (Certification No. NK4.)
5. Safranin O, ca. 0.5% in half xylene, half 100% alcohol. (Certification No. NS-11.)
6. Methylene blue (chloride), ca. 0.5% in half xylene, half 100% alcohol. (Certification No. NA-12.)
7. Other general stains may serve.

Mesophyll (General Stains)

1. Foster's tannic-acid-iron-chloride (see Johansen, 1940, p. 91).
2. Fast green FCF, ca. 0.5% in 100% alcohol. (Certification No. NGf-6)
3. Congo red, ca. 1% aqueous or weak alcohol. Grüber.
4. Bismarck brown Y, ca. 1% in 100% alcohol. (Sample purchased from University Apparatus Co., Berkeley, Calif.)
5. Aniline blue, ca. 1% in 95% alcohol. (Certification No. NK-4 used)

6. Brilliant green, *ca.* 0.5% in 100% alcohol. (Certification No. NBg-13.)
7. Light green SF, *ca.* 0.5% in 100% alcohol. (Certification No. NL-9.)
8. Erythrosin, *ca.* 1% in 100% alcohol. (Harmer Laboratories Co.)

Sclerenchyma and Tracheary Elements

1. Safranin O, *ca.* 0.5% in half xylene, half 100% alcohol. (Certification No. NS-11.)
2. Methylene blue (chloride), *ca.* 0.5% in half xylene, half 100% alcohol. (Certification No. NA-12.)
3. Acid fuchsin, *ca.* 0.5% in 70% alcohol. (Certification No. NR-19.)
4. Crystal violet, *ca.* 0.5% in 100% alcohol. (Certification No. NC-21.)
5. Delafield's hematoxylin, overstained and de-stained in acid alcohol, 20% to 50% aqueous.
6. Bismarck brown Y, *ca.* 1% in 100% alcohol. (Sample purchased from University Apparatus Co., Berkeley, Calif.)
7. Iodine green, *ca.* 1% in 100% alcohol.

As can be seen, many of the above dyes stain approximately the same structures that they do in uncleared material, although usually not so well. The epidermis in particular stains poorly. It is possible that some of the microchemical tests may prove useful as stains, although few were tried by the writer.

In general, difficulties caused by rapid leaching are surmounted by overstaining high up in the dehydration series, destaining briefly in the same solvent, and moving through to xylene. If the dye is in water solution, the material must be run through as rapidly as possible. Twenty to thirty minutes staining is generally sufficient staining time for all the dyes listed, depending of course on the thickness of the material. The concentrations and solvents given can be varied widely. Destaining is often complete in fifteen minutes, although fast green, Congo red, acid fuchsin, and aniline blue tend to leach out more slowly than the rest. Rapidity of destaining and degree of differentiation depend on the material used; some dyes may not destain at all in certain cases. For most of the stains, removal can be accelerated with acidified alcohol. This includes tannic-acid-iron-chloride. If destained too much, the material can usually be replaced in the dye with no ill effects. Delafield's and Harris' hematoxylin require the use of acidified alcohol for destaining, but the stain is removed first from the cuticle and epidermis. Therefore these

regions will not show well if destaining is carried out. The Congo red can be improved by staining in about a 0.2% aqueous solution, moving to 95% alcohol, acidifying slightly with HCl until the dye turns dark purplish black, rinsing in 100% alcohol, and moving on up the series. Variability is great; staining qualities of leaves from different species and of leaves from different plants of the same species may be entirely different, and correct staining therefore becomes a matter of individual treatment.

Double staining is often advantageous. Stevens (1916, p. 302) recommended the use of Bismarck brown preceded by safranin. Two other workers have kindly permitted me to mention their procedures. Dr. O. T. Bonnett of the University of Illinois, working with cleared primordia of corn ears and tassels, has found a combination of Bismarck brown Y and fast green FCF most suitable for bringing out the thin-walled cells of the meristematic region and at the same time maintaining the high transparency needed with his thick material. He stains the material five to ten minutes in a 1% solution of Bismarck brown in 95% ethyl alcohol, destains in the same strength alcohol, stains one to two minutes in a 1% solution of fast green in 100% alcohol, and then if necessary gives the material a second treatment in a 1% solution of Bismarck brown in 100% alcohol to replace what dye the surface tissues may have lost. Dr. A. S. Foster, of this institution, investigating cleared leaves of *Quina* (Quinaceae), is successfully using tannic acid-iron chloride for a general stain, followed by safranin to give a contrasting background and to bring out the procambium and tracheary elements. Other combinations may likewise prove valuable.

Brownish pigments remain after clearing some materials, and must be bleached out. The writer, using Stockwell's fluid for this purpose (Johansen, 1940, p. 85), found that the subsequent staining was sometimes unaffected, sometimes improved, and other times was made unsatisfactory. The subject of bleaches, their effects, and the use of compounds to restore staining capacity, needs study.

Different clearing compounds may also affect staining, although the general uses of the different dyes will probably remain the same. For instance, material bleached in Chlorox and stained with safranin takes a more general stain than material cleared in NaOH, destains much more slowly, and at the same time differentiates the vascular elements more poorly. For various reasons the chloral hydrate (Stevens, 1916, pp. 299, 301), lactophenol (Klebahn, 1910), lactic acid (Simpson, 1929; Sporne, 1948), hydrogen peroxide with ammonium hydroxide (Stebbins, 1938; Sporne, 1948), sodium hypochlorite (McVeigh, 1935; Gabriel and Pellisier, 1936), and potassium hypo-

chlorite (Eau de Javelle: Stevens, 1916, p. 307) methods proved unsatisfactory for the material used here, and therefore the writer has only limited experience with them. Dr. Bonnett, however, has found that while lactic acid itself will not sufficiently clear his material, it improves clearing when used after NaOH, and at the same time improves the staining of his combination of Bismarck brown with fast green. Other stains as well may be made more effective. This leads to the whole question of mordants, another field which has not been explored with regard to cleared material. The writer had some success with iodine as a mordant for crystal violet and with Delafield's hematoxylin as one for methyl green, and it is probable that further use of mordants will yield worthwhile improvements in staining.

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DETERIORATION OF SCHIFF'S REAGENT

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ABSTRACT.—Deterioration of Schiff's (Feulgen's) reagent was studied under varying conditions. Photometric readings were taken of both induced and spontaneous color development and curves which showed the rate of deterioration were plotted. It was concluded that Schiff's reagent, when kept in well-filled and tightly corked bottles at 0°–5°C., will retain staining efficiency for at least six months.

This study was undertaken to observe accurately the deterioration of Schiff's (Feulgen's) reagent under varying conditions of time and temperature.

Essentially two factors are involved in its deterioration. One is an irreversible color change caused by exposure to air, and the other is a partly reversible change, caused by a shifting of chemical equilibrium between the sulfite and dye (Karrer, 1946). Fresh Schiff's reagent sealed in air-tight tubes will, upon exposure to temperatures of 60°C., rapidly take on a deep violet color. If the tubes are then cooled, the color disappears. This reversibility is still demonstrable after 24 hours at 60°C. After approximately 48 hours at 60°C., however, the coloration by heat tends to become permanent and not completely removed by cooling. Both the irreversible (oxidative) and the partially reversible (heat) changes in the reagent were observed under controlled conditions.

Schiff's reagent was prepared (de Tomasi, 1936) by dissolving 0.5 g. of basic fuchsin by pouring over it 100 ml. of boiling distilled water. The mixture was shaken thoroughly, allowed to cool to 50° C., and filtered; 10 ml. of 1N HCl followed by 0.5 g. of potassium metabisulfite ($K_2S_2O_5$) were added to the filtrate. The solution was allowed to stand in the dark, well stoppered, overnight. Residual color was removed (Coleman, 1938) by adding 0.25 g. of activated charcoal, shaking and filtering.

Equal amounts of solution were placed in glass stoppered bottles and stored at temperatures of 60°, 40°, 25°, 15° and 0° C. The ability of these solutions to develop color was checked weekly by

¹Publication number 509 from the Department of Anatomy, Northwestern University Medical School.

means of a photometer. One-half milliliter of reagent, 1 ml. of 10% formalin, and 20 ml. of distilled water were placed in a test tube, and the color which developed in one-half hour was read photometrically against a distilled water standard, using a red (650 $m\mu$) filter. The results are shown in Fig. 1. A photometric reading of 2 indicates

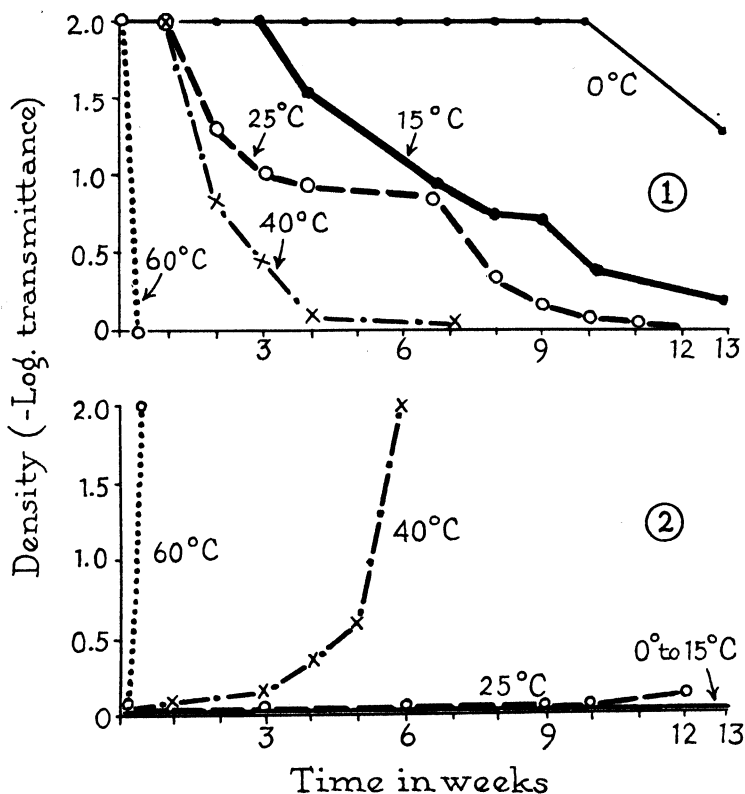


FIG. 1.—Amount of color developed by Schiff's reagent when formaldehyde was added after varying periods of storage at various temperatures.

FIG. 2.—Amount of color developed spontaneously by Schiff's reagent on storage at various temperatures.

the maximum color development possible by freshly prepared Schiff's reagent in an excess of formalin. The Coleman charcoal method was used to remove any recolored dye that appeared spontaneously through the process of deterioration before taking the readings.

The curves in Fig. 1 show that deterioration is very rapid at 60° or even 40°, but slow between 0°–5°. This supports Lillie's (1947) suggestion that Schiff's reagent should be stored at temperatures from 0° to 5°.

At intervals, tissues were stained with the solutions by the Picro-Feulgen block method (Lhotka and Davenport, 1947) to determine the histological efficiency of the reagents. A photometric reading 0.669 coincided with the first histological evidence of deterioration in the solutions, and any reagent that had deteriorated to this point was considered to be unsatisfactory for staining.

The reversible, spontaneous production of color by heat was studied by placing 20 ml. of reagent in sealed test tubes. These tubes were stored at the same temperatures as the bottles. They were checked weekly for spontaneous color development by reading them photometrically in the same way that the formalin-developed color was read. No formalin was added to these tubes, and they remained sealed throughout the experiment. The findings are shown in Fig. 2. These curves show that heat deterioration is rapid at 60° but negligible below 25°. The initial reversibility of this reaction was shown, in that solutions at 60° which had developed a photometric reading of .824 were returned to a colorless state by cooling below 25°.

From these studies, it can be concluded that Schiff's reagent may be prepared for the Feulgen stain in stock quantities provided it is stored at temperatures of 0° to 5°C. and is contained in well filled, tightly stoppered bottles. The curves which show the course of its deterioration indicate that it will retain its staining efficiency for at least six months and probably longer.

ACKNOWLEDGMENT

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MOUNTING MEDIA FOR PHASE MICROSCOPE SPECIMENS

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ABSTRACT.—Results with phase microscopy can often be improved by the proper selection of mounting media. Temporary mounts can first be made in liquid mixtures of water and glycerol, butyl carbitol and alpha-chloronaphthalene, alpha-chloronaphthalene and alpha-bromonaphthalene or alpha-bromonaphthalene with methylene iodide to determine what index liquid serves best to emphasize structures of primary interest. Once this has been determined, permanent mounts can be prepared by the substitution of a solid mounting medium that most closely approaches the refractive index of the liquid.

Many papers have been published with reference to the superiority of phase microscopy over the usual brightfield and dark-field methods for the examination of living zoological and botanical specimens. Applied to paraffin sections of tissue, whole mounts or sections of textile fibers, and chemicals and minerals, phase microscopy has not always produced spectacular results, and in a few cases bright-field illumination has even been preferred. Barer (1947) states that for fixed neurological material phase microscopy is sometimes at a disadvantage. Too much detail is often rendered visible when a selective effect is desired, as is possible with the use of specific stains. Some of these apparent failures are due to the use of specimens that are too thick, incorrect cover-glass thickness when using the $43\times(4\text{ mm.})$ 0.65 N. A. objective, and the employment of mounting media of unsuitable refractive index. The choice of index liquids or solids for mounting is somewhat dependent on the relative refractive index of constituents within the preparation. For optimum results, objects of primary interest within the specimen should appear dark against a lighter background. This effect with positive contrast objectives can be obtained when the object of chief interest has a slightly higher refractive index than its background and the mounting medium employed is slightly below the object of interest in refractive index. It is the purpose of this paper to suggest a series of liquids and solid mounting media, some of which have been employed in brightfield and dark-field microscopy, that might be used to improve results with phase microscopy.

MOUNTING MEDIA FOR LIVING PREPARATIONS

The primary characteristic of mounting media for living preparations is that they must not cause plasmolysis or plasmoptysis of the cell. Such isotonic media are either the liquids in which the cell lives or are artificially prepared solutions. Specific examples are protozoa or bacteria mounted in their culture media, blood cells in their serum or normal saline, and epithelial cells of the mouth in saliva. In all cases the mounting media consist mainly of water having an index of $n_D=1.33$ or slightly higher, dependent on the amount of material in solution. A survey of the literature reveals that little information is available as to the refractive index of the living cell. Lee (1937) makes the statement that its index is slightly higher than water. Maximow and Bloom (1930) give the index of the lens of the eye as 1.36 in the peripheral layers and 1.42 in the inner zone or nucleus, where the consistency of the tissue is harder. From this information it would seem that these isotonic liquids for the living cell, being slightly below the index of most cell constituents, would be quite ideal for producing good contrast. In general this has proved to be true. For example, if an epithelial cell from the mouth is examined in saliva ($n_D=1.33+$) with positive contrast phase objectives, the nucleus and most of the cytoplasmic granules, having a slightly higher index than the saliva, appear dark against the lighter background of the ground substance. The results for some structures within this preparation and for certain constituents of other living histological specimens might possibly be improved by the substitution of liquids of slightly higher index as mounting media. The difficulty involved in preparing any such liquids is to find an aqueous solution that has a higher index and at the same time is isotonic.

MOUNTING MEDIA FOR NON-LIVING PREPARATIONS

With reference to non-living preparations we are not limited to isotonic solutions and thus a larger number of mounting media are available. The question, however, is raised as to what index liquid or solid will provide the maximum contrast for certain structures within a textile fiber, inclusions within a crystal, mitochondria within a cell, chromatin in the nucleus, etc. This can be determined by the preparation of a series of index liquids as suggested for the dispersion staining method (Crossmon, 1948, 1949) ranging in index from about 1.33 to 1.70 and differing in index by 0.004. The specimen is temporarily mounted in these media to determine what index will give the greatest contrast to the object of primary interest. For an

index range of 1.33 to 1.436, mixtures of distilled water with glycerol are suggested; for 1.440 to 1.628, diethylene glycol monobutyl ether (butyl carbitol) and alpha-chloronaphthalene; for 1.632 to 1.656, alpha-chloronaphthalene and alpha-bromonaphthalene; and for 1.660 to 1.70, alpha-bromonaphthalene with methylene iodide. The quantity of the lower index liquid to be mixed with the higher to give the intermediate indices can be determined by the formula:

$$V_1n_1 + V_2n_2 = V_xn_x$$

where V represents volume, n the index and V_xn_x the volume and index desired. Before substitution of the index value for the lower and higher constituents in the formula, it is best to accurately determine their index by means of a refractometer at 25°C. To obtain 10 ml. of a 1.520 index liquid as is used as a mounting medium for the section of prostate gland, a mixture of 5.3 ml. of butyl carbitol with 4.7 ml. of alpha-chloronaphthalene would be required, as shown in the computation below:

$$\begin{array}{r}
 1.430V_1 + 1.620V_2 = 10(1.520) \\
 V_1 + \quad \quad V_2 = 10 \text{ ml.} \\
 1.430V_1 + 1.620V_2 = 15.20 \\
 1.430V_1 + 1.430V_2 = 14.30 \\
 \hline
 0.190V_2 = 0.90 \\
 V_2 = 4.7 \text{ ml.} \\
 V_1 = 5.3 \text{ ml.}
 \end{array}$$

For more accurate results than determined by the formula, the index of the liquids thus prepared should be checked on a refractometer at 25°C. and corrections made by adding the necessary quantity of the lower or higher index liquid.

The refractive indices of minerals, chemicals, textile fibers and plastics can be found in chemical handbooks or published papers, and from that information an idea can be formed as to the range of index liquids that should be used as mounting media. Little information is available concerning the refractive indices of fixed histological specimens. Our work with paraffin sections of formaldehyde-fixed tissue of the white rat indicates that many histological structures are slightly below or above 1.536. According to Manly (1938), dental structures are considerably higher. In a review of the literature, he finds that enamel has been reported as having an index of 1.627 for the ordinary ray and 1.6234 for the extraordinary ray. The index of normal dentine is given as 1.577 ± 0.003 . Secondary cementum, according to his own investigation, varies in index from 1.560 to 1.570.

The procedure for temporary mounting of histological preparations is as follows. Paraffin microtome sections of suitable thickness (6–10 μ) of zoological or botanical material or of textile fibers should be attached to clean slides by the water and desiccation method (Crossmon, 1948) and after drying placed in xylene to remove the paraffin. In preparation for mounting, the xylene surrounding the section should be wiped away with a lint-free cloth and the section gently blotted face down against blotting paper. A drop of one of the index liquids (about 1.520 is suggested as a trial for formaldehyde-fixed tissue) is placed on the preparation and a cover-glass applied. The thickness of the cover-glass used is very important when using the 43 \times (4 mm.) 0.65 N.A. objective. Best results are obtained when its thickness is close to 0.18 mm. Should a preparation as a whole or constituents within the preparation appear white, the index liquid used is either much lower or higher than the object. An illustration is to first mount a microtome section in water ($n_D=1.33$) and after dehydrating remount in methylene iodide ($n_D=1.74$). In both cases all cell constituents will appear white with little or no detail. However, as we substitute liquids closer to the index of the specimen, structures slightly above the liquid will appear dark and much greater contrast will be evident.

The index liquids suggested are only temporary mounts. For permanent preparations the cover-glass must be ringed with cement or solid mounting media must be substituted. Let us suppose that a certain structure in a neurological preparation gives the best contrast in a 1.534 liquid and that we wish to have a permanent preparation. A reference to the list of solid mounting media given in this paper gives the index of solid Canada balsam as about 1.53. This should prove to be a suitable substitute. Consideration must be made that its solvent such as xylene lowers its index and it is not until the solvent has evaporated that the index of solid balsam is attained. Also, balsam, as well as other natural resins, varies slightly in index dependent on the method of preparation.

Other solid mounting media that have been used in microscopical technic or have been suggested in the literature are given in the table below. Detailed information concerning their preparation, properties or source can be found in the references named. The refractive indices given are as stated in the literature cited. They should be accepted with some caution, since for the most part no information is given as to whether the index stated represents the solid or solid dissolved in a solvent.

The photographs of unstained prostate gland reproduced in this publication are typical of the different contrasts that can be obtained

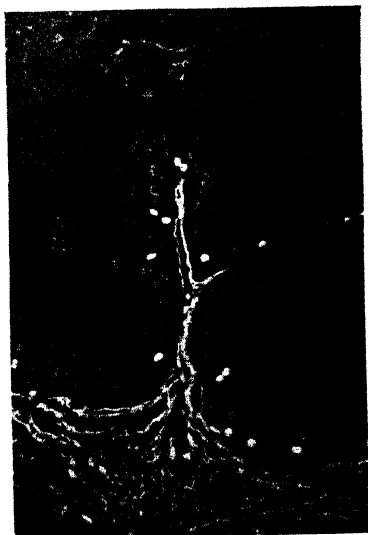
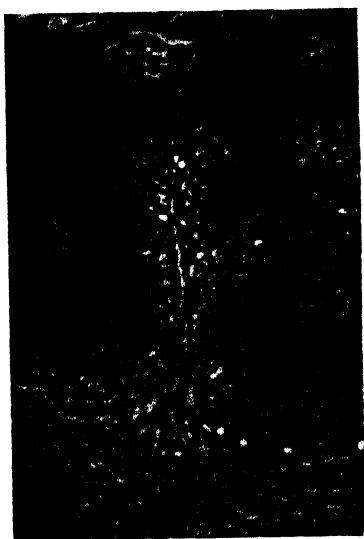
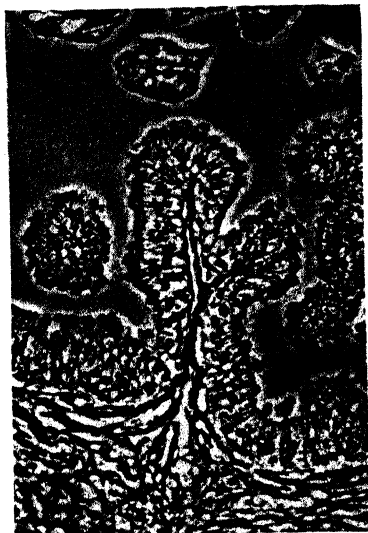
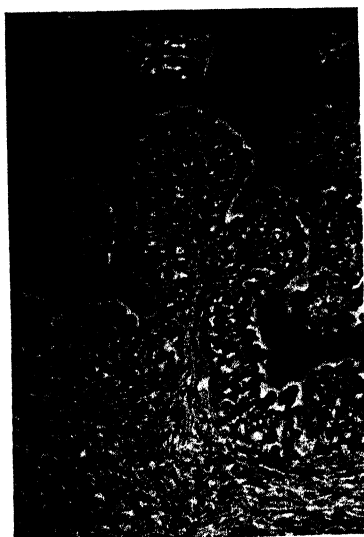


FIG. 1-4.—All photographs are of exactly the same area of a 10μ section of human prostate gland fixed in formalin, the only difference being the index of the mounting media (butyl carbitol with alpha-chloronaphthalene). Mag. $300\times$ using the $21\times$ (8 mm.) 0.50 N.A. objective.

FIG. 1.—Refractive index of mounting medium n_D 1.430.

FIG. 2.—Refractive index of mounting medium n_D 1.508.

FIG. 3.—Refractive index of mounting medium n_D 1.520.

FIG. 4.—Refractive index of mounting medium n_D 1.548

dependent on the mounting medium employed. Fig. 1 shows a section mounted in a liquid of n_D 1.430, considerably lower in index than the majority of the tissue constituents. Most of the tissue elements are white and little contrast is evident. The mounting media employed in Fig. 2 and 3 are both almost ideal dependent on what

TABLE 1. REFRACTIVE INDEX OF VARIOUS MOUNTING MEDIA

Name	Refractive Index	Reference
Glychrogel A	1.41 (solid)	Stain Techn., 10, 21-2. 1935.
Isobutyl methacrylate	1.47	Shillaber, C. P. Photomicrography, p. 498. 1944.
Glycerin jelly	1.48	Shillaber, C. P. Photomicrography, p. 497. 1944.
Sandarac camphloral	1.485	Lee, A. B. The Microtometist's Vade-Mecum, p. 228. 1937.
Camsal balsam	1.485	Lee, A. B. The Microtometist's Vade-Mecum, p. 227. 1937.
Aroclor and Isobutyl methacrylate	1.495-1.525 (solid)	Text. Res. J., 18, 756-8. 1948.
Dammar	1.52	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Canada balsam	1.53 (solid)	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Euparal	1.53 (solid)	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Clarite	1.54 (solid)	Shillaber, C. P. Photomicrography, p. 502. 1944.
Clarite X	1.57 (solid)	Shillaber, C. P. Photomicrography, p. 502. 1944.
European styrax	1.582	Corrington, J. D. Working with the Microscope, p. 207. 1941.
American styrax	1.63	Corrington, J. D. Working with the Microscope, p. 207. 1941.
Aroclor	1.63	Shillaber, C. P. Photomicrography, p. 503. 1944.
Styrene resin	1.66	Chamot, E. M., and Mason, C. W. Handbook of Chemical Microscopy, p. 167. 1938.
Piperine	1.681	Corrington, J. D. Working with the Microscope, p. 207. 1941.
Hyrax	1.71	Corrington, J. D. Working with the Microscope, p. 207. 1941.
Glychrogel B	1.75 (solid)	Stain Techn., 10, 21-2. 1935.
Pleurax	1.75	J. Roy. Micr. Soc., 69, 25-8. 1949.
Naphrax	1.76	J. Roy. Micr. Soc., 63, 34-7. 1943.
Sirax	1.8	Corrington, J. D. Working with the Microscope, p. 207. 1941.

tissue structures are of primary interest. Note especially the dark connective tissue in Fig. 2 and the white structures (probably nuclei of much lower or higher refractive index than the mounting medium) in Fig. 3. Fig. 4 represents the effect obtained when mounting medium of too high an index is employed. Note that the connective tissue shown as dark in Fig. 2 is light in this photograph.

ACKNOWLEDGMENT

The writer wishes to thank Mr. Louis Schrader for his assistance in preparing the photographs and index liquids necessary for the completion of this work.

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NOTES ON TECHNIC

A SUGGESTION FOR PREVENTION OF LOOSE SECTIONS IN THE BODIAN PROTARGOL METHOD

Prolonged immersion in an aqueous medium such as the protargol bath in the original Bodian method for paraffin sections may increase any tendency for sections to separate from the slide during their subsequent reduction.

Even though all usual precautions against loosening have been taken we find that losses from detached sections are often suffered, particularly if the object is large and of heterogeneous character (e.g. the kidney) and therefore difficult to flatten properly.

For those who still prefer the longer immersion time of the original method to shorter modifications the following simple suggestion should be helpful.

Acidifying the rinse which precedes reduction in sodium sulfite and hydroquinone with as little as 0.5 ml. of 2% acetic acid to 50 ml. of water greatly reduces losses from sections which might otherwise loosen. We have tested this suggestion repeatedly for a period of several months and find that detached sections rarely result and that the differentiation of the staining is improved as well.—HELEN DAVIES and PINCKNEY J. HARMAN, *Department of Anatomy, College of Medicine, New York University.*

THE DIFFERENTIAL STAINING OF AMPHIBIAN YOLK GRANULES

Sections of frog ova and embryos can be stained in an aqueous solution of thionin, and then treated with alcoholic eosin; as a result the yolk is differentiated into basophilic and eosinophilic granules. The procedure is as follows:

1. Specimens are fixed in Smith's modification of Tellyesnick's fluid for 24 hours, washed in running water for six hours, and then passed through the alcohols (30%, 50%, 70%, 83%, and 95%), remaining in each for 20–30 minutes; fresh 95% alcohol for 30 minutes follows.

2. Clearing in cedarwood oil for two hours is followed by immersion in xylene for three to five minutes; treatment with melted paraffin (m.p. about 52°C.) for three to five hours follows, during which time the paraffin is changed once.

3. Embedding, sectioning, and affixation to slides is carried out in the usual manner.

4. Paraffin is removed from the sections by placing the slides in xylene for three minutes.

5. Immersion in absolute alcohol for one minute is followed by passage through the alcohols (95%, 70%, 50%, and 30%), allowing thirty seconds in each.

6. The slides are rinsed in water for fifteen seconds, then placed in a saturated aqueous solution of *thionin* (C.I. No. 920) for one to two hours. After this the slides are rapidly rinsed in water, and all excess water is carefully wiped off.

7. Material is immersed in 95% alcohol for 30 seconds, then stained with 0.5% alcoholic *Eosin Y* (C. I. No. 768) for 15–30 seconds.

8. Treatment with 95% alcohol for 30 seconds is followed by mounting in green Euparal.

This technic clearly differentiates the large vegetal yolk granules (blue) from the small granules (red) making up the remainder of the yolk. It may therefore be of value to investigations concerned with tracing the disposition and utilization of the yolk granules in various stages of amphibian development.

Stock solutions of the stains are prepared by dissolving 0.5 g. of eosin in 100 ml. of 95% alcohol, and by dissolving 0.25 g. of thionin in 100 ml. of distilled water; the stains used by the author were manufactured by the National Aniline Division, Allied Chemical and Dye Corp.—IRA J. LAUFER, *New York University, New York, N. Y.*

LABORATORY HINTS FROM THE LITERATURE

DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS
DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

HILLIER, J. Some remarks on the image contrast in electron microscopy and the two-component objective. *J. Bact.*, 57, 313-7. 1949.

The introduction of a weak lens component between the specimen and the conventional electron microscope lens has increased the working distance of the objective ten times though the focal length is essentially unchanged. Bacteria photographed with the new objective show considerably more internal structure than was revealed with the conventional objective. However, both spherical and chromatic aberrations are also increased.—V. Kavanagh.

DYES AND THEIR BIOLOGICAL USES

PACKARD, C. E. The effects of certain chemicals on the macronucleus of *Spirostomum teres*, with notes on the genus. *Trans. Amer. Micr. Soc.*, 67, 275-9. 1948.

Safranin and methyl green in varying concentrations were used as vital stains. The macronuclei showed considerable variation in size and shape in different concentrations of these dyes.—V. Kavanagh.

ANIMAL MICROTECHNIC

ARCADI, J. A. Horteaga's silver carbonate method applied to gill parasites; with a note on the head organs. *Trans. Amer. Micr. Soc.*, 67, 285-9. 1948.

Silver technics seem never before to have been applied to gill flukes but gave exceptionally clear pictures. The method was applied as follows: Fix parasites freed of mucus in 1% formalin for 4 days; transfer the parasites with a capillary pipette to concentrated NH_4OH for 3-4 min.; wash thoroughly in 2 changes of distilled water; impregnate in the prepared ammoniacal silver solution for 30 sec.—8 min. (50 sec. optimum); wash in 2 changes distilled water; reduce in 1% formalin for 30 sec.; wash as above; fix in 1% $\text{Na}_2\text{S}_2\text{O}_3$ a few sec.; wash in distilled water; mount in glycerin-gelatin medium as modified by Mizelle and Seamster (*J. Parasitol.*, 25, 501-7, 1939). The ammoniacal silver solution which gave best results was prepared as follows: 5 ml. 10% AgNO_3 , 20 ml. 5% Na_2CO_3 , concentrated NH_4OH to dissolve the precipitate and distilled water to make 75 ml.—V. Kavanagh.

BRECHER, GEORGE. The structure of unstained reticulocytes. *Proc. Soc. Exp. Biol. and Med.*, 69, 89-90. 1948.

Examination of a wet film of mouse blood with a phase microscope revealed an occasional red cell which contained a delicate reticulum. Adding hypotonic potassium or ammonium oxalate to the blood increased the number of cells which showed intracytoplasmic structures, particularly rods and granules. Smears that were dried and fixed in alcohol failed to show these cytoplasmic structures. Dried smears mounted in 10% formalin or 1.2% ammonium oxalate showed structures similar to those seen in the fresh preparations. The identity of the cells which contained rods and granules with reticulocytes could be demonstrated by staining with a small amount (concentration not stated) of brilliant cresyl blue or of new methylene blue added to the oxalate solution. Human reticulocytes contained rods and granules similar to those seen in the mouse.—H. A. Davenport.

FIGGE, FRANK H. J., WEILAND, G. S., and MANGANIELLO, L. O. J. Cancer detection and therapy. Affinity of neoplastic, embryonic, and traumatized tissues for porphyrins and metalloporphyrins. *Proc. Soc. Exp. Biol. and Med.*, 69, 640-41. 1948.

Hematoporphyrin in 1 mg. doses was injected intraperitoneally into tumor

bearing and non-tumor-bearing mice. Sacrifice at 24-hour intervals showed that most of the porphyrin migrated to the tumors in 24-48 hr. Porphyrin was demonstrated by its fluorescence, and its presence in tissues other than tumors found to be limited to the great omentum and lymph nodes. Tumors of uninjected tumor bearing mice were negative for hematoporphyrin. Other porphyrins (protoporphyrin, mesoporphyrin and coproporphyrin) showed the same tendency to be concentrated in neoplastic tissue, but other fluorescent substances (fluorescein, rhodamine, dihydro-collidine and riboflavine) did not. A zinc-porphyrin compound was concentrated by tumors. The possibility of using radioactive porphyrin in the therapy of lymphatic leukemia and in the detection of deep cancer is suggested.—H. A. Davenport.

ISENBERG, H. D. Simplified method for staining spermatozoa. *Amer. J. Clin. Path.*, 18, 94. 1949.

The following modification of Meaker's staining procedure is described as a simple method of obtaining clear pictures of spermatozoan morphology: Shake manually at least 5 minutes 1 part of seminal fluid with 1 part of 5% NaHCO₃; centrifuge 2-3 minutes at 2000-3000 R.P.M.; pour off supernatant; wash centrifuge in normal saline corresponding to original volume and recentrifuge; repeat saline washing; add a few drops of saline to centrifuge and prepare smear with loop as for bacterial stain; dry at room temperature or at 37°C. in incubator, and fix 2-3 sec. with heat; flush slide with 95% ethanol, drain and allow to dry; stain 3 min. with a 1:1 mixture of carbol-fuchsin (no details) and 95% ethanol; wash in water, and treat 2 min. with a 1:3 aqueous methylene blue solution (no details); rinse at least 1 min. in running water, dry without blotting. The spermatozoa head-caps stain light blue, the nuclear posterior portion of the heads dark blue, and the body and tail portions light red or pink.—L. Farber.

KITZMILLER, J. B. The use of dioxane in insect microtechnique. *Trans. Amer. Micr. Soc.*, 67, 226-30. 1948.

The use of dioxane simplified the process of sectioning aphids. The method is as follows: Bouin's fluid, 1 hr. to several days; dioxane, 1 hr.; fresh dioxane, overnight; Tissuemat, 2 hr. to overnight; embed in 56-58° Tissuemat. The longer times were advantageous except for aphids in the first or second instars. The aphids were handled in shallow gauze-bottomed dishes made from 1 in. glass tubing cut in ¾ in. sections to which gauze was tied tightly. These dishes were set in Stender dishes of the reagents. Embedding was done in the sloping molds suggested by Solberg.—V. Kavanagh.

PLANT MICROTECHNIC

BROADFOOT, H. H., and SCHWARZ, E. R. An improved permanent mounting medium for textile fibers. *Textile Res. J.*, 18, 756-8. 1948.

Permanent colorless mounting media for textile fibers can be prepared by mixing together a synthetic resin (isobutyl methacrylate, np 1.48) with a plasticizer, Aroclor 1242, np 1.62, and dissolving the mixture in xylene to a viscosity close to that of balsam. By varying the proportion of each constituent it is possible to adjust the refractive index to a value suitable for a particular fiber. Mixtures having a refractive index of between 1.495 and 1.520 are suggested for cotton and wool, and between 1.515 and 1.520 for acetate rayon. The procedure for mounting is essentially the same as for Canada balsam.—G. Crossmon.

MICROÖRGANISMS

LORD, THOMAS H., and SMULL, MARGARET M. An improved microscopic method of examining fatty foods. *Food Res.*, 14, 241-2. 1949.

An improved method for making smears of fatty foods for microscopic examination for bacteria is outlined as follows: (a) Melt the sample of oleomargine at 45°C. (113°F.) within a 15-min. period. Shake thoroughly. (b) Place 0.1 ml. of the sample on a standard glass slide and add one drop of Mayer's solution and three drops of a 1% "Tide" solution. (c) Mix vigorously with an "L"-shaped inoculating needle and then smear over the entire area of the slide. (d) Fix quickly by passing the slide through the flame of the Bunsen burner three times. This is done rapidly so that the oil droplets will not coalesce into large areas but

will remain minute and evenly distributed over the slide. (e) Drip xylene over the slide to remove the fat, then immerse the slide in 95% alcohol from 30 to 40 sec. Dry in air. (f) Again drip xylene over the smear and dry in the air. Repeat until the smear is "cleared"; then dry. (g) Stain with Loeffler's alkaline methylene blue stain for about 30 sec. (h) Wash by dipping the slide into two jars of water. Allow to dry at room temperature. (i) Examine under the oil immersion lens, the field of which has been standardized to a diameter of 0.157 mm. The average number of organisms in 100 fields multiplied by 1,000,000 equals the number of organisms per milliliter of melted sample.

The improved appearance of the finished smear and the greater consistency of results led the authors to submit this method for general use by others. Although "Tide" is not a pure chemical compound, its general availability and low cost made it seem a desirable ingredient to use, especially under field conditions. Any other surface-active agent or detergent should prove equally useful, provided it does not cause disruption of the cells or alter the staining properties of the smears.—*William. G. Walter.*

MAY, J. Zur fluoreszenzmikroskopischen Unterscheidung lebender und toter Bakterien mittels Akridinorangeaufarbeitung. *Zentbl. Bakt., 1 Abt. Orig.*, 152, 586-90. 1948.

Varied fluorescent-microscopical examinations of 18 different microorganisms, stained with acridine-orange were performed. This was done to test Strugger's claim that living and dead bacteria can be differentiated by their color reaction. The author disclaims the validity of Strugger's theory. He found that the green, orange, and red color reflex of bacteria depends on concentration and pH of the dye solutions. The color scheme does not allow differentiation between living and dead bacteria.—*Iran Saphra.* (Courtesy *Biological Abstracts*).

MICHAEL, PAUL, and MARKELL, E. K. Modified Papanicolaou stain for amebae. *Amer. J. Clin. Path.*, 19, 81. 1949.

A combination of the iron hematoxylin stain with the Papanicolaou stain used as a counterstain is reported to improve the accuracy in differentiating amebae from other cells, such as histiocytes, to enable cytopathologic changes to be demonstrated. The recommended technic is as follows: Prepare smears as usual; before they have become dry, fix for 30 min. in freshly prepared Schaudinn's fluid containing 5% glacial acetic acid; rinse thoroughly in 70% ethanol, and treat 5 min. with 95% ethanol containing enough Gram's iodine to produce a port wine color; rinse thoroughly in 70% and 50% ethanol and in tap water; mordant 4 min. in 4% ferric ammonium sulfate; rinse in tap water; stain 10 min. in 0.5% aqueous hematoxylin; rinse in tap water; destain 12 min. in 0.25% ferric ammonium sulfate (made fresh daily from 4% stock solution), wash 5 min. in running tap water and rinse in distilled water; rinse successively in 50, 70, and 95% ethanol; stain 3 min. in OG6; rinse in 2 changes of 95% ethanol; stain 6 min. in EA 50; rinse in 3 changes of 95% ethanol followed by absolute ethanol; transfer to xylene; and mount in balsam or clarite.—*L. Farber.*

NICHOLS, M. STARR. Gentian (crystal) violet solution for Gram stain. *Amer. J. Clin. Path.*, 18, 98. 1949.

The recommended solution is prepared as follows: Dissolve 2 g. oxalic acid in 880 ml. of water, add 20 ml. of aniline and shake to dissolve; dissolve 5 g. gentian (crystal) violet in 100 ml. of 95% ethanol and mix with oxalic-acid-aniline solution. It is claimed that this solution stains Gram-positive organisms well, and not any of the Gram-negative ones so far tested, and that it has good keeping qualities without developing any precipitate.—*L. Farber.*

PENNINGTON, DERROL. The use of periodate in microbiological staining. *J. Bact.*, 57, 163-7. 1949.

Polysaccharides may be demonstrated by a periodate oxidation to form aldehyde groups which may be demonstrated by Schiff's reagent. The method is as follows: Fix bacteria by heat, in Bouin's solution, or other fixative; immerse them in 1% aqueous solution of sodium metaperiodate for 5-15 min. at room temperature; wash thoroughly with water; stain 15 min. in sulfite-decolorized basic fuchsin (Coleman, *Stain Techn.*, 13, 123-4, 1938); wash 10 min. in SO₂

water (5 ml. 10% $K_2S_2O_8$ and 5 ml. 0.1 N HCl in 100 ml. water); wash in water; dry. Metachromatin or volutin granules in spirilla and diphtheria bacilli disintegrate with the periodate.—V. Kavanagh.

RAWSON, ARNOLD J. Acid-fast property of *Histoplasma capsulatum*. *Amer. J. Clin. Path.*, 18, 97. 1949.

Tissue sections have been stained with the following modification of the Ziehl-Neelsen method with a larger number of organisms retaining the carbol-fuchsin stain than with the usual technic. Instead of using acid alcohol, 3% aqueous hydrochloric acid is used to decolorize the stained sections, which are then stained 30 sec. with methylene blue and passed rapidly through 95% and absolute ethanol before being placed in xylene. As with other staining procedures, only a variable number of the forms present were acid-fast.—L. Farber.

WEINREBE, N. Stain for *Borrelia recurrentis*. *Amer. J. Clin. Path.*, 18, 99. 1949.

It is claimed that the following modification of Giemsa's staining technic for *B. recurrentis* gives more distinct preparations than the usual one. The thick drop smear (as for malarial stain) is dried without fixation in methyl alcohol and stained 1 hour in a mixture of 2 drops Giemsa's stain and 1 ml. of distilled water.—L. Farber.

HISTOCHEMISTRY

BOYD, GEORGE A., and WILLIAMS, AGNES I. Stripping film technics for histological autoradiographs. *Proc. Soc. Exp. Biol. and Med.*, 69, 225-32. 1948.

Nuclear track emulsion stripping film (Eastman Kodak Co. NTB) was used successfully for autoradiography of histological sections. Such film, with a 10 μ cellulose ester base and a 10 μ thickness of emulsion, permitted mounting the section on either the emulsion side or the cellulose-base side. A variation in the distance between the section and the emulsion was advantageous in that it facilitated the identification of the actual cell or other histologic structure which contained the radioactive tracer. By mounting the section on the cellulose-base side of the film, developing the emulsion could be accomplished without subjecting the section to deleterious actions of the photographic developing and fixing solutions. Illustrations are given of the alpha particle "sunbursts" and the exact histologic site from whence the particle came. Artifacts caused by accidental abrasion of the emulsion and by the process of stripping are discussed.—H. A. Davenport.

CARRERA, G. M., and CHANGUS, G. W. Demonstration of acid phosphatase in *Endamoeba histolytica*. *Proc. Soc. Exp. Biol. and Med.*, 68, 610-11. 1948.

Acid phosphatase activity was shown by Gomori's method. Smears containing *E. histolytica* were allowed to dry at room temperature until they became "glossy" but not completely dry, and fixed by immersion in chilled acetone for 30 sec. A positive reaction was obtained, however, on smears stored 26 hr. in acetone in the refrigerator. Incubation in the reaction mixture at room temperature (26-29° C.) 45 min. to 2 hr. sufficed to give a deposit of phosphate in the amebae. Negative results were obtained when the glycerophosphate was omitted from the incubation mixture or if NaF (M/300) was added to it. Two figures illustrate the stained specimen and an unstained control.—H. A. Davenport.

DOYLE, WILLIAM L. Effects of dehydrating agents on phosphatases in the lymphatic nodules of the rabbit appendix. *Proc. Soc. Exp. Biol. and Med.*, 69, 43-4. 1948.

The loss of activity of both acid and alkaline phosphatases was studied after the following methods of preservation: absolute acetone and 80% acetone, absolute and 80% ethyl alcohol, absolute methyl alcohol, and quick freezing followed by drying *in vacuo*. The activity of freshly homogenized tissue in terms of phenol liberated from disodium phenyl phosphate in a glycine or barbiturate buffer was optimum for alkaline phosphatase at pH 9.8 and was 7-11 μ g. of phenol per milligram of fresh tissue during 30 min. at 30° C. Acid phosphatase was

most active at pH 5.6–5.8 and liberated 10–14 μ g. of phenol under the same conditions of time and temperature. Alkaline phosphatase activity of fresh tissue was found to be reduced to about 75% of the fresh value after fixation for 24 hr. in either absolute acetone, 80% acetone or absolute alcohol. After 80% ethyl alcohol, the activity was found to be about 65%. Only about 15% remained after fixation in absolute methyl alcohol. Acid phosphatase showed 25% of its original activity after acetone, 15% after ethyl alcohol, and a negligible amount after methyl alcohol. Better cytological fixation and preservation of enzymes were obtained when the tissue was dropped into acetone at -20° C. and stored at this temperature for 48 hr. Acetone-fixed material came through paraffin embedding with 60% acid, and 55% alkaline phosphatase activity. Phosphatases were stable at room temperature for several days in the block but deteriorated in ribbons. However, ribbons stored at -20° C. kept their activity for a week.—*H. A. Davenport.*

GOMORI, G. Histochemical demonstration of sites of choline esterase activity.
Proc. Soc. Exp. Biol. and Med., **68**, 354–8. 1948.

The procedure recommended is as follows: Fix and dehydrate tissue in ice-cold acetone. Transfer to a 1:1 mixture of absolute alcohol and ether for 1–3 hr., then into a 4% solution of collodion in alcohol-ether for 12 hr. while still in the refrigerator. Pass through two 1-hour changes of chloroform at room temperature and embed in paraffin. Cut sections and mount as usual, remove paraffin and bring to distilled water. Incubate the slides in a buffered solution of choline ester for 2–16 hr. at 37° C. The buffer stock solution is made by dissolving 29 g. of maleic acid and 30.4 g. of tris (hydroxy-methyl)-aminomethane in 500 ml. of water, decolorizing with a few grams of charcoal and filtering. To 10 ml. of this stock solution, 12 ml. of 0.5N NaOH is added (pH 7.7) and the mixture diluted to 50 ml. The working solution is made by adding 30 ml. of 0.1M cobaltous acetate diluted to 300 ml. with distilled water to the pH 7.7 buffer and then adding about 1 mg. each of CaCl_2 , MgCl_2 and MnCl_2 . The choline ester is prepared as a 0.02M solution in distilled water. The ester solution is added to the completed buffer mixture in the proportion of 1 ml. ester to 50 ml. of buffer at 37 – 40° C. Myristoyl choline ester is recommended as the most satisfactory. Lauroyl, palmitoyl, and stearoyl choline esters were tried also. After incubation, the slides are washed in tap water and then immersed in a dilute solution of light yellow ammonium sulfide (a few drops of the concentrated reagent to 50 ml. of distilled water) for 15 min. They are then washed under the tap, counterstained as desired, dehydrated, cleared and covered. The sites of choline esterase activity are stained black. Five figures are given which show esterase activity in the nerve cells of the brain of the mouse, the conducting system of the heart in the dog, spindle structures in striped muscle of the mouse, and the reactions to two different substrates of the sympathetic ganglia of the rabbit. The reaction is inhibited in most tissues by 0.00001M prostigmine.—*H. A. Davenport.*

GOMORI, G. Histochemical demonstration of sites of phosphamidase activity.
Proc. Soc. Exp. Biol. and Med., **69**, 407–9. 1948.

In a study of the histochemical specificity of phosphatases (Gomori, *Proc. Soc. Exp. Biol. and Med.*, **70**, 7–11, 1949—following abstract), one substrate out of 19 gave a reaction at pH 5 which indicated the existence of a specific enzyme, a “phosphamidase”. The unique substrate, p-chloranilidiphosphonic acid, was processed to yield the purified, free phosphonic acid, and this compound was used as a substrate with histologic sections to determine the sites of phosphamidase activity. The following procedure is recommended: Fix thin slices of tissue in chilled acetone, or in 95% or absolute alcohol, and make paraffin sections therefrom. Deparaffinize, bring to water, and incubate in substrate-buffer mixture. Prepare the buffered mixture by taking 50 ml. of 0.05 M maleate buffer, pH 5.6 (5.8 g. maleic acid plus 62 ml. of N NaOH in 1000 ml.), adding thereto 1.0–1.5 ml. of 0.1 M $\text{Pb}(\text{NO}_3)_2$ solution, a few drops of 10% solution of MnCl_2 , and shaking until the precipitate dissolves. Add 2 ml. of the phosphonate stock solution made by dissolving a calculated amount of the purified phosphonic acid in an excess of 10% NH_4OH , adjusting to pH 8 with acetic acid, and bringing up to volume with distilled water. Place the staining mixture in an oven at 45 – 60° C. until the $\text{Pb}_3(\text{PO}_4)_2$ settles. Filter into a Coplin jar which is subsequently inclined at an angle of 30° and place the slides face downward in it.

After incubation for 10–24 hr. at 37–40°, rinse the slides in distilled water and wipe precipitate from the backs of the slides and around the tissue. Remove the remaining superficial precipitate by placing the slides in a 0.1 *M*. citrate buffer, pH 4.5–5.0, and rinse thoroughly under the tap as soon as the slide appears to be completely clear around the tissue. (This differentiation is the most critical step in the procedure since insufficient treatment may leave precipitate which appears black in the finished stain and overtreatment may remove the enzymatic reaction product.) Treat the slides with a dilute solution of yellow ammonium sulfide (about 10 drops of the concentrated reagent to the Coplin jar). Wash in tap water, counterstain if desired, dehydrate, clear and cover. Treatment for 10 min. in Lugol's solution or in any 1 *N* mineral acid inactivates the enzyme and permits the making of control slides. Small amounts of the enzyme are present in many normal tissues and large amounts are found in the gray matter of the central nervous system and in malignant epithelial tumors.—*H. A. Davenport*.

GOMORI, G. *Histochemical specificity of phosphatases.* *Proc. Soc. Exp. Biol. and Med.*, 70, 7–11. 1949.

The problems of substrate specificity, organ specificity and effects of specific activators and inhibitors are discussed briefly. The experimental data were obtained by the application of histochemical technic. Six to 20 different tissues, fixed in chilled alcohol or acetone were embedded in a single paraffin block. Sections were mounted on slides and incubated 1–24 hr. with the substrate to be tested. The following substrates were used: metaphosphate; pyrophosphate; methyl-, glycerol-, and aminoethyl phosphate; phenyl, *o*-chlorophenyl, α -naphthyl-, resorcylic- and phenolphthalein phosphate; glucose-1-phosphate; hexosediphosphate; adenosinetriphosphate; yeast nucleate; thymonucleate; phosphoryl choline and lecithin; octanoyl-phosphate; and *p*-chloranilidophosphonate. The concentrations of substrates ranged from 0.02 to 0.005 *M* or, in the case of nucleates and lecithin, from 0.1 to 0.5%. Experiments were performed at pH5, pH7 and pH9. The cation used to trap the phosphate ions was Ca at pH9 and Pb at pH5 and 7. Pyrophosphate gave no positive reaction for enzymatic hydrolysis, and metaphosphate reacted only on the surface of intestinal villi at pH9. Some nonspecific staining was observed, but when this was deleted, the distribution of enzyme was the same for all substrates. The only exception was *p*-chloranilidophosphonate which gave a different distribution of enzyme in the acid range. Among the other 18 substrates which gave positive reactions, there was no evidence of phosphatases other than the common non-specific acid and alkaline variety, and the pattern of distribution of enzymatic activity in any given organ was constant and independent of the substrate used.—*H. A. Davenport*.

MEYER, R. L. *A new chromosome stain and its relationship to atypical cell proliferation.* *Proc. Soc. Exp. Biol. and Med.*, 68, 664–8. 1948.

Salivary glands of *Drosophila* were used as test material. The stain was prepared by dissolving 0.2 g. paraphenylenediamine in 20 ml. of hot 20% acetic acid, adding 0.1 ml. of 30% H₂O₂, and cooling the mixture as soon as a light brown color developed. Oxidation was allowed to continue for about 30 min. before use. To make the stained preparation, isolated glands were suspended on a slide with a drop of 50% acetic acid (they may be pulped to facilitate staining). Contamination with iron from dissecting needles should be avoided. The slides were stained as follows: Blot off the acetic acid and apply the staining solution. Allow to remain in a covered Petri dish over moist filter paper for 1 hr. Apply a cover glass and make squash preparation in the usual way. A second method suggested was to dissolve 10 mg. of quinone diimine crystals in 1 ml. of 70% acetic acid and apply to the material to be stained. The stain develops in 10–20 min. Chromosomes become dark purple-brownish while cytoplasm remains almost unstained. The *s*-dimethyl- and tetramethyl paraphenylenediamine as well as quinone chlorimine and quinone dichlorimine did not stain. Chromosomes stained with quinone diimine resembled those stained with aceto-carmin, aceto-orcin and the Feulgen technic. It is suggested that the formation of the stain is due to the fixation of the staining agent by the tissue as an analido-quinone type of compound, which involves nucleoprotein. Attention is called to the relationship of this reaction to atypical epithelial proliferations and cancer formation which are caused by certain aromatic amines.—*H. A. Davenport*.

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